January 2018

Identification and characterization of farR and farE as a regulator and effector of fatty acid resistance in Staphylococcus aureus

Heba Alnaseri
The University of Western Ontario

Supervisor
McGavin, Martin J.
The University of Western Ontario

Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

© Heba Alnaseri 2017

Follow this and additional works at: https://ir.lib.uwo.ca/etd
Part of the Bacteriology Commons

Recommended Citation
https://ir.lib.uwo.ca/etd/5131

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact tadam@uwo.ca, wlswadmin@uwo.ca.
Abstract

Although *Staphylococcus aureus* is exposed to antimicrobial fatty acids on the skin, in nasal secretions and in abscesses, specific mechanisms for regulating gene expression and intrinsic resistance in response to these fatty acids have not been reported. Through *in vitro* selection for increased resistance of *S. aureus* to linoleic acid, I identified fatty acid resistant clone FAR7, where a single nucleotide polymorphism caused a His\textsubscript{121}Tyr substitution in an uncharacterized member of the TetR family of transcriptional regulators, which is divergently transcribed from a gene encoding a member of the resistance-nodulation-division superfamily of multi-drug efflux pumps. I named these genes *farE* and *farR*, for regulator and effector of fatty acid resistance, respectively. *S. aureus ΔfarER* exhibited loss of inducible resistance to linoleic acid, and although FarR is a TetR family regulator which typically repress expression of a divergent gene, I found that FarR is needed to induce *farE*. Compared to wild type *S. aureus*, FAR7 exhibited increased expression of *farR* and *farE* under non-inducing conditions, and a significantly higher induced level of *farE*. Electrophoretic mobility shift assays revealed a FarR binding site in the *farER* intergenic segment, that overlaps with the +1 transcription start site of *farR* as determined by 5′-RACE. The variant FarR7 produced by *S. aureus* FAR7 failed to bind to this operator site, and nucleotide substitutions within the operator abolished binding of native FarR. Conversely, FarR and FarR7 bound equally well to a second operator site upstream of the predicted *farE* promoter. Therefore, like other TetR regulators, FarR represses its own expression, and a His\textsubscript{121}Tyr substitution in FarR causes a loss of auto-repression and increases expression of both *farR* and *farE*. My data reports the first description of a specific mechanism of inducible resistance to antimicrobial fatty acids in a Gram-positive pathogen and defines a new paradigm for regulation of a divergently transcribed gene by a TetR family regulator.

Keywords

Antimicrobial fatty acids, efflux pumps, TetR family regulator, inducible resistance, *Staphylococcus aureus.*
Co-Authorship Statement

All studies presented in this thesis were completed by Heba Alnaseri in the laboratory of Dr. Martin McGavin with assistance from co-authors as listed below. Dr. Martin McGavin contributed to the conceptualization, design, data analysis and manuscript preparation of all experiments.

**Chapter 2: Inducible expression of a resistance-nodulation-division-type efflux pump in *Staphylococcus aureus* provides resistance to linoleic and arachidonic acids.**

This chapter has been previously published:


Benjamin Arsic selected the fatty acid resistant clones that were sent for comparative genome sequencing. James Schneider and Zachariah Scinocca assisted in the *farE* reporter gene assays. Julie Kaiser assisted with the set-up of [*\textsuperscript{14}C*]linoleic acid uptake assays.

**Chapter 3: Identification of FarR binding site responsible for auto-repression and efflux-mediated resistance to antimicrobial fatty acids in *Staphylococcus aureus*.**

This chapter is being prepared for submission to a peer-reviewed journal.

Katherine A. Ferguson assisted with *farR* reporter gene assays and western blotting. James Schneider constructed the USA300ΔfakA mutant and helped with *farE* reporter gene assay in this background.
Epigraph

“You may encounter many defeats, but you must not be defeated. In fact, it may be necessary to encounter the defeats, so you can know who you are, what you can rise from, how you can still come out of it.”

-Maya Angelou
Dedication

To the remarkable woman who gave up on her dreams so I can have mine; this is for you mom.
Acknowledgements

To my supervisor Dr. Martin McGavin: I owe my deepest gratitude to you for your mentorship, patience and encouragement throughout the course of my doctoral studies. Thank you for always challenging me and pushing me to think critically and boost my intellectual curiosity. I am truly fortunate to have be given the opportunity to learn from you, grow and become an independent researcher. None of this would have ever possible without your generosity, and for everything you have done for me Dr. McGavin, I will be forever in your debt.

To Dr. David Heinrichs and Dr. John McCormick: I would like to sincerely thank you for the time you devoted towards helping me. Your guidance, accessibility and kind support have been essential for my graduate studies. I am truly grateful for your engaging conversations during my committee meetings. You helped foster my confidence in myself as a scientist in more ways than you can ever imagine. To the Heinrichs and McCormick lab members: I would like to thank you for always being willing to listen to me, share your resources and for always making me feel welcomed. It was a true pleasure to have shared great conversations and plenty of laughter with all of you.

To McGavin Lab members: I would like to thank you for fostering an incredibly supportive and enjoyable environment to work in. Thank you for hearing me vent about failed experiments, and sharing my joy about successful ones. Thank you for being there for me every time I needed to brainstorm, and helping me cover up my occasional “secret science” from start to finish, whether it was successful or not. To Piraveina Gnanasuntharam, James Schneider, Zachariah Scinocca, Jamie Halucha, Robert Kuiack and Katherine Ferguson, I thank you for your support at every lab meeting, journal club, seminar, and conference that I have ever attended. More importantly, I thank you all for your genuine friendship over the years. This journey would have never been the same without each and every single one of you.
To the department of Microbiology and Immunology faculty, staff and students: I would like to thank you for the kindness you have shown me over the years. I could not have asked for a more supportive environment to grow personally and academically. To Elizabeth Coley and Fernanda Russell: I would like to especially thank you for your endless support and encouragement during the past five years. I sincerely will miss you both very much.

To mom, dad, Mody and Oreo: words cannot express how grateful I am for your unconditional love and support. I thank you for your patience and understanding throughout my lengthy education. I thank you for never doubting my ability to face any challenges even when I had all the reasons to doubt myself. Thank you for always having my back; none of this would mean anything without you. Mom: I am beyond grateful to you for the values and strong work ethics you instilled in me. I hope you know that everything I am today or will ever hope to be I owe it to you.
# Table of Contents

Title Page ........................................................................................................................................... i
Certificate of Examination .................................................................................................................. ii
Abstract .................................................................................................................................................... iii
Co-Authorship Statement ...................................................................................................................... iv
Epigraph ................................................................................................................................................... v
Dedication ................................................................................................................................................ vi
Acknowledgments .................................................................................................................................. vii
Table of Contents ................................................................................................................................. ix
List of Tables .......................................................................................................................................... xiii
List of Figures .......................................................................................................................................... xiv
List of Appendices .............................................................................................................................. xvi
List of Abbreviations ............................................................................................................................ xvii
Chapter 1 ............................................................................................................................................... 1
  1 Introduction ..................................................................................................................................... 1
    1.1 *Staphylococcus aureus* Overview ............................................................................................... 2
    1.2 Emergence of Methicillin-Resistant Community-acquired *Staphylococcus aureus* .................. 2
    1.3 *Staphylococcus aureus* colonization ........................................................................................... 3
    1.4 *Staphylococcus aureus* pathogenesis and clinical manifestation ............................................. 4
    1.5 Genetic regulation of *Staphylococcus aureus* virulence ............................................................. 10
    1.6 Antimicrobial Resistance mechanisms of *Staphylococcus aureus* ......................................... 14
    1.7 *Staphylococcus aureus* envelope architecture .......................................................................... 15
      1.7.1 Cell envelope composition ..................................................................................................... 15
      1.7.2 Membrane homeostasis ........................................................................................................ 16
1.8 Fatty acid machinery of *Staphylococcus aureus* ........................................ 22
   1.8.1 Phospholipid composition ...................................................................... 22
   1.8.2 Phospholipid synthesis ........................................................................... 22
   1.8.3 Lipids as antimicrobials ........................................................................... 28
1.9 Bacterial efflux pumps .................................................................................. 34
1.10 TetR family of transcriptional regulators and their biological functions .......... 35
1.11 Rationale and hypothesis ............................................................................ 37
1.12 Literature cited ............................................................................................ 40

Chapter 2 ............................................................................................................ 65

2 Inducible expression of a Resistance-Nodulation-Division-Type efflux pump in *Staphylococcus aureus* provides resistance to Linoleic and Arachidonic Acids .... 65
2.1 Introduction .................................................................................................... 66

2.2 Materials and methods .................................................................................. 68
   2.2.1 Bacterial strains and growth conditions .................................................. 68
   2.2.2 Selection and comparative genome sequencing of fatty acid resistant (FAR) clones .......................................................... 71
   2.2.3 Strain and plasmid construction ............................................................... 72
   2.2.4 Assay of growth and bactericidal activity ............................................... 75
   2.2.5 Assay for uptake of $^{14}$C-linoleic acid ................................................... 75
   2.2.6 farE::lux reporter gene assays ................................................................. 76
   2.2.7 Data analyses ........................................................................................ 76
2.3 Results ............................................................................................................ 77
   2.3.1 Identification of single nucleotide polymorphism in linoleic acid resistant variants of *S. aureus* ................................................................. 77
   2.3.2 Description of the farE-farR locus ............................................................ 77
   2.3.3 farR is required for inducible resistance to linoleic acid ......................... 78
2.3.4 *farE* contributes to persistence and growth of *S. aureus* in the presence of linoleic acid................................................................. 82

2.3.5 The FAR7 clone exhibits increased expression of *farE*................................. 87

2.3.6 An H₁₂₁Y substitution in FarR is sufficient for increased resistance to linoleic acid................................................................. 87

2.3.7 Role of *farE* in resistance to other uFFA........................................... 90

2.3.8 Inactivation of *farE* promotes increased uptake of ¹⁴C-linoleic acid....... 96

2.4 Discussion.................................................................................................. 99

2.5 References............................................................................................... 104

Chapter 3...................................................................................................... 112

3 Identification of FarR binding sites responsible for auto-repression and efflux-mediated resistance to antimicrobial fatty acids in *Staphylococcus aureus* ................ 112

3.1 Introduction.............................................................................................. 113

3.2 Materials and methods............................................................................. 114

3.2.1 Bacterial strains and growth conditions.............................................. 114

3.2.2 Construction of *S. aureus* USA300Δ*farER* and USA300Δ*fakA* ........... 115

3.2.3 Construction of complementation and reporter gene constructs......... 116

3.2.4 RNA isolation and 5′-rapid amplification of complementary DNA ends ................................................................. 121

3.2.5 Construction and purification of recombinant FarR............................ 121

3.2.6 FarR-DNA interaction studies............................................................. 122

3.2.7 Antibody production and western blotting ......................................... 123

3.2.8 Murine infection model........................................................................ 123

3.2.9 Computer analyses............................................................................... 124

3.3 Results..................................................................................................... 124

3.3.1 Deletion of *farER* results in loss of inducible resistance to linoleic acid 124

3.3.2 *farR* is required for *farE* induction................................................. 125
3.3.3 Identification of farR promoter and transcription start site .......... 128
3.3.4 Expression of farR is subject to auto-regulation ...................... 128
3.3.5 Identification of FarR operator sites .................................. 132
3.3.6 FarR binds OP_{Act} and OP_{Rep} .................................. 137
3.3.7 OP_{Rep} contains the site of farR auto-repression and a His_{121}Tyr substitution in FarR causes relief of auto-repression ............. 139
3.3.8 Specificity determinants of FarR binding ................................ 144
3.3.9 farER and fatty acid detoxification ..................................... 150
3.3.10 farER influence virulence in a subcutaneous abscess infection model... 153

3.4 Discussion ................................................................................. 155
3.5 References .............................................................................. 161

Chapter 4 ......................................................................................... 165

4 General Discussion and Conclusions ........................................... 165
4.1 Summary .................................................................................. 166
4.2 Limitations and future studies .................................................. 168
4.3 References .............................................................................. 176

Appendices ..................................................................................... 178

Curriculum Vitae .............................................................................. 186
List of Tables

Table 1.1 Summary of *S. aureus* virulence determinants. ........................................ 6
Table 1.2 Major regulators of virulence determinants in *S. aureus*. ............................. 12
Table 1.3 Summary of proposed mechanisms of uFFA toxicity. .................................. 30
Table 1.4 Summary of uFFA resistance mechanisms proposed to date. ........................... 33
Table 2.1 Strains and plasmids used in Chapter 2. ...................................................... 69
Table 2.2 Primers used for construction of plasmids in Chapter 2. ............................... 74
Table 3.1 Strains and plasmids used in Chapter 3. ....................................................... 117
Table 3.2 Oligonucleotides used in Chapter 3. ............................................................. 119
List of Figures

Figure 1.1 Regulation of lipid homeostasis in E. coli ................................................................. 17
Figure 1.2 Regulation of lipid homeostasis in B. subtilis ............................................................. 21
Figure 1.3 Overview of fatty acid and phospholipid biosynthesis in S. aureus .................. 26
Figure 1.4 Overview of exogenous fatty acid utilization by S. aureus ................................. 27
Figure 2.1 Linoleic acid induces expression of farE ................................................................. 80
Figure 2.2 Sensitivity of USA300 and USA300farR::ΦNE to the bactericidal activity of 100 µM linoleic acid ................................................................. 81
Figure 2.3 Mutation of farE::ΦNE enhances sensitivity of S. aureus to toxicity of linoleic acid ................................................................. 85
Figure 2.4 Sensitivity of USA300 and USA300farE::ΦNE cells to the bactericidal activity of 100 µM linoleic acid ................................................................. 86
Figure 2.5 The FAR7 SNP causes enhanced induction of farE expression ..................... 88
Figure 2.6 FAR7 is more resistant than USA300 to linoleic acid ......................................... 89
Figure 2.7 The variant farR7 allele, but not wild type farR, enables the ability of USA300farR::ΦNE to grow at inhibitory concentrations of linoleic acid ................................. 92
Figure 2.8 Influence of different antimicrobial fatty acids on induction of farE, or viability of S. aureus USA300 and USA300farE::ΦNE ................................................................. 94
Figure 2.9 Effect of farE::ΦNE and Δtet38 mutations on growth of S. aureus in the presence of 25 µM or 40 µM palmitoleic acid (PA) ................................................................. 95
Figure 2.10 Growth (A) and uptake of 14C-linoleic acid (B) following exposure of S. aureus USA300 and USA300farE::ΦNE to an increase in concentration of linoleic acid ................................................................. 98
Figure 3.1 farER contributes to resistance of S. aureus USA300 to linoleic acid ....... 126
Figure 3.2 farR is required for farE induction ................................................................. 127
Figure 3.3 Nucleotide sequence of farER intergenic segment showing PfarR and PfarE promoter features, and farR::lux reporter gene assays conducted in E. coli and S. aureus ................................................................. 131
Figure 3.4 FarR operator sites are located in the farER intergenic segment ..................... 136
Figure 3.5 Competition EMSA with OP2 reveals that an operator site for binding of FarR is located within OP13 .......................................................... 138

Figure 3.6 FarR and the variant FarR7 differ in binding to OP2 and OP13, both of which span core promoter elements of \( P_{farR} \) .......................................................... 143

Figure 3.7 OP4, OP5 and OP13 cross-compete with each other and nucleotide substitutions obliterate this competition. ......................................................... 146

Figure 3.8 Multiple sequence alignment of the intergenic segment between farE and farR among \( S. aureus \) USA300 and 12 other staphylococcal species that contain divergent \( farER \) .......................................................... 147

Figure 3.9 Nucleotide substitution in the -10 element of \( P_{farR} \) causes a relief of auto-repression. .................................................................................. 149

Figure 3.10 farE::lux exhibits elevated constitutive levels of expression in the absence of fatty acid kinase, fakA .......................................................... 151

Figure 3.11 USA300ΔfakA exhibits enhanced resistance to linoleic acid, in a farE-dependent manner .......................................................... 152

Figure 3.12 USA300ΔfakA-farER exhibits reduced virulence in a murine skin abscess model of infection .......................................................... 154

Figure 3.13 FarR binding to \( OP_{Rep} \) is partially affected by linoleoyl-CoA and arachidonoyl-CoA .......................................................... 160

Figure 4.1 Schematic model of the potential mechanism of \( P_{farE} \) regulation by FarR, depending on exogenous fatty acid ligand .......................................................... 172

Figure 4.2 Secondary structure prediction of farE and farR RNA using RNAfold Webserver .......................................................... 175
List of Appendices

Appendix 1. ASM Journals Statement of Author’s Rights.................................................. 178

Appendix 2. Adaptation of S. aureus USA300 during growth in TSB + 50 µM linoleic acid.......................................................... 179

Appendix 3. Differential effect of pGYlux and pGYfarE::lux on growth of S. aureus USA300 in TSB + 20 µM linoleic acid. .......................................................... 180

Appendix 4. Exponential phase cells are highly susceptible to the bactericidal activity of 100 µM linoleic acid (LA). .......................................................... 181

Appendix 5. Cadmium inducible expression of farR protects USA300farR::ΦNE from the bactericidal activity of 100 µM linoleic acid. .............................. 182

Appendix 6. FAR7, but not USA300, is able to grow in TSB containing 50 µM palmitoleic acid (PA). .......................................................... 183

Appendix 7. fakA-farER influence virulence in a murine skin abscess model of infection. .......................................................... 184

Appendix 8. Purification of 6×His-tagged FarR from wildtype USA300 and the variant FAR7 using nickel affinity chromatography. .................................................. 185
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ</td>
<td>Delta for deletion</td>
</tr>
<tr>
<td>ΔfakA</td>
<td>fakA deletion</td>
</tr>
<tr>
<td>ΔfarER</td>
<td>farER deletion</td>
</tr>
<tr>
<td>Δtet38</td>
<td>tet38 deletion</td>
</tr>
<tr>
<td>ΔfakA-ΔfarER</td>
<td>fakA and farER double deletion</td>
</tr>
<tr>
<td>α toxin</td>
<td>Alpha toxin</td>
</tr>
<tr>
<td>β-defensin</td>
<td>Beta defensin</td>
</tr>
<tr>
<td>β hemolysin</td>
<td>Beta hemolysin</td>
</tr>
<tr>
<td>β-lactamase</td>
<td>Beta lactamase</td>
</tr>
<tr>
<td>δ toxin</td>
<td>Delta toxin</td>
</tr>
<tr>
<td>γ toxin</td>
<td>Gamma toxin</td>
</tr>
<tr>
<td>µCi</td>
<td>microcurie</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µL</td>
<td>microlitre</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
</tr>
<tr>
<td>µm</td>
<td>micrometre</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>xg</td>
<td>times gravity</td>
</tr>
<tr>
<td>5’-RACE</td>
<td>5’- rapid amplification of complementary DNA ends</td>
</tr>
<tr>
<td>AAP</td>
<td>Abridged anchor primer</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ACME</td>
<td>Arginine catabolic mobile element</td>
</tr>
<tr>
<td>Acr</td>
<td>Acriflavine resistance</td>
</tr>
<tr>
<td>agr</td>
<td>accessory gene regulator</td>
</tr>
<tr>
<td>AmtR</td>
<td>Regulator of ammonium uptake</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUAP</td>
<td>Abridged universal amplification primer</td>
</tr>
<tr>
<td>bp</td>
<td>basepair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Carbon</td>
</tr>
<tr>
<td>CA-MRSA</td>
<td>Community-acquired methicillin-resistant Staphylococcus aureus</td>
</tr>
<tr>
<td>Cd</td>
<td>Cadmium</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>Cm</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>cme</td>
<td>Campylobacter multidrug efflux gene</td>
</tr>
<tr>
<td>DDT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>Dha</td>
<td>Dihydroxyacetone</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>Erm</td>
<td>Erythromycin</td>
</tr>
<tr>
<td>EthR</td>
<td>Ethionamide repressor</td>
</tr>
<tr>
<td>fab</td>
<td>Fatty acid biosynthetic gene</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Fad</td>
<td>Fatty acid degradation</td>
</tr>
<tr>
<td>Fak</td>
<td>Fatty acid kinase</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty acid modifying enzyme</td>
</tr>
<tr>
<td>FapR</td>
<td>Fatty acid and phospholipid biosynthesis regulator</td>
</tr>
<tr>
<td>FAR</td>
<td>Fatty acid resistance</td>
</tr>
<tr>
<td>FarE</td>
<td>Effector of fatty acid resistance</td>
</tr>
<tr>
<td>FarR</td>
<td>Regulator of fatty acid resistance</td>
</tr>
<tr>
<td>FarR7</td>
<td>FarR protein from the variant FAR7 clone</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthesis</td>
</tr>
<tr>
<td>G3P</td>
<td>Glycerol-3-phosphate</td>
</tr>
<tr>
<td>Geh</td>
<td>Glycerol ester hydrolase</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglicosamine</td>
</tr>
<tr>
<td>GSP</td>
<td>Gene specific primer</td>
</tr>
<tr>
<td>HA-MRSA</td>
<td>Hospital-associated methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>H</td>
<td>Histidine</td>
</tr>
<tr>
<td>HapR</td>
<td>Regulator of HA/protease gene in <em>Vibrio cholerae</em></td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>Ica</td>
<td>Intercellular adhesion</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl 1-thio-β-D-galactopyranoside</td>
</tr>
<tr>
<td>IR</td>
<td>Imperfect repeat</td>
</tr>
<tr>
<td>IRDye</td>
<td>Infrared dye</td>
</tr>
<tr>
<td>Isd</td>
<td>Iron-regulated surface determinant</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>LA</td>
<td>Linoleic acid</td>
</tr>
<tr>
<td>Lac</td>
<td>Los Angeles County clone</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>Luk</td>
<td>Leukocidins</td>
</tr>
<tr>
<td>LysR</td>
<td>Lysine regulator</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>ManNAc</td>
<td>N-acetylmannnosamine</td>
</tr>
<tr>
<td>Mar</td>
<td>Multiple antibiotic resistance</td>
</tr>
<tr>
<td>MATE</td>
<td>Multidrug and toxic compound extrusion</td>
</tr>
<tr>
<td>MFS</td>
<td>Major facilitator superfamily</td>
</tr>
<tr>
<td>Mg^2+</td>
<td>Magnesium ion</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MLST</td>
<td>Multi-locus sequence type</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MMPL</td>
<td>Mycobacterium membrane protein large</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>Manganese (II) chloride</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MSA</td>
<td>Mannitol Salt Agar</td>
</tr>
<tr>
<td>MSCRAMMs</td>
<td>Microbial surface components recognizing adhesive matrix molecules</td>
</tr>
<tr>
<td>MSSA</td>
<td>Methicillin-susceptible <em>Staphylococcus aureus</em></td>
</tr>
</tbody>
</table>
Mtr  Multiple transferable resistance
MurNAc  N-acetylmuramic acid
NaCl  Sodium chloride
ns  Non-significant
OD  Optical density
OP  Operator
OP_{Act}  Activation operator site
OP_{Rep}  Repression operator site
P  Promoter
P_{farE}  farE promoter
P_{farR}  farR promoter
PA  Palmitoleic acid
PAGE  Polyacrylamide gel electrophoresis
PAL  Pseudo-palindrome
PBP2a  penicillin-binding protein 2a
PBS  Phosphate-buffered saline
PCR  Polymerase chain reaction
PHYRE  Protein homology/analogy recognition engine
Pls  Phosphate acyltransferase
pmol  picomole
poly[d(I-C)]  Poly-deoxyinosinic-deoxyctydyllic acid
psi  Pound-force per square inch
PtdIns  Phosphatidylinositol
PVDF  Polyvinylidene difluoride
PVL  Panton-valentine leukocidin
Qac  Quaternary ammonium antiseptic compounds
RBS  Ribosomal binding site
RLU  Relative light units
RND  Resistance nodulation cell division
rot  Repressor of toxin
RPM  Rotation per minute
RutR  Regulator of pyrimidine utilization operon
sar  Staphylococcal accessory regulator
sae  S. aureus exoprotein
SCCmec  Staphylococcal cassette chromosome
SczA  Streptococcus zinc-sensing regulator
SdiA  Regulator that suppresses cell division inhibitors
SDS  Sodium dodecyl sulfate
SMR  Small multidrug resistance
SNP  Single nucleotide polymorphism
SoxS  Superoxide response regulon transcriptional activator
SspA  Staphylococcus serine protease
T7SS  Type VII secretion system
TBE  Tris-borate-EDTA
Tet  Tetracycline
TFR  TetR family of transcriptional regulators
TSA  Tryptic soy agar  
TSB  Tryptic soy broth  
TSS  Transcription start site  
Tyr  Tyrosine  
uFFA  Unsaturated free fatty acid  
UTR  Untranslated region  
V   Volt  
Y   Tyrosine
Chapter 1

1 Introduction
1.1 *Staphylococcus aureus* Overview

*Staphylococcus aureus* is a Gram-positive bacterium from the Firmicutes phylum that is considered as a pathobiont. It colonizes ~30% of the human population asymptomatically, and thus is a part of the resident microbiota, but also a pathogen proficient in causing diverse cutaneous and systemic infections of ranging severity (1). In his attempt to examine the underlying cause of blood-poisoning in 1881, Alexander Ogston observed micrococci with a spherical outline that stained a uniformly deep violet and grouped into clusters. He examined a series of chronic and acute abscesses and found them to contain micrococci mingled with pus. When these micrococci were injected into subcutaneous tissue of test animals, they were capable of proliferating, forming inflammatory knots, invading the tissue, and disseminating into the blood stream. In 1882, these micrococci were named *Staphylococcus* (2–4). Since this first description as a causative agent of human infection, *S. aureus* has been and continues to be a leading cause of human infectious morbidity and mortality. Over the past few decades, community-acquired methicillin-resistant *S. aureus* (CA-MRSA) strains have become a significant disease burden on healthcare systems worldwide (5). These strains display hyper-virulence and effective host-to-host transmission at a relatively minor fitness cost, posing a serious threat to public health systems (6). In fact, MRSA accounts for 78% of the skin and soft tissue infections presented to emergency departments in eleven cities in the United States; 98% of these infections being caused by the community-acquired MRSA (CA-MRSA) clone USA300, which is the strain of choice in our studies (7).

1.2 Emergence of Methicillin-Resistant Community-acquired *Staphylococcus aureus*

Prior to the debut of penicillin, the mortality rate of *S. aureus* bacteremia patients was as high as 80% (8), and as early as two short years after the introduction of the antibiotics, resistant strains of *S. aureus* resistant began to emerge (9). Penicillin-resistant *S. aureus* isolates, owing to the production of β-lactamases, were reported in the healthcare and community settings in the 1950s (10). Subsequently, methicillin use was introduced into clinical practice, but the resilient *S. aureus* developed methicillin resistance rapidly (11, 12). MRSA infections represent a significant burden on the healthcare system, and are
found to be associated with increased length of hospitalization (13, 14). In a survey of staphylococcal infections across the United States, Canada, Latin America, Europe, and the Western Pacific region, MRSA was found to be the most prevalent cause of bacteremia, pneumonia, and skin and soft tissue infections (15). For decades, MRSA infections were mostly limited to healthcare settings such as hospitals and nursing homes; however, in the late 1990s community-acquired isolates were first reported. The prevalent clinical presentations were mainly fatal necrotizing pneumonia, septicemia, and pulmonary abscesses (16, 17). There were five CA-MRSA strains responsible for the majority of staphylococcal disease worldwide; the Midwest Clone, the Southwest Pacific Oceania Clone, the European Clone, the Pacific Clone, and the USA300 clone (18). USA300 was named after the unique profile of its pulse-field gel electrophoresis (19). This clone which has been responsible for severe disease outbreaks in the past (17, 20), is currently the predominant strain of S. aureus in North America, and accounts for 98% of infections presented to emergency departments (7, 21).

1.3 Staphylococcus aureus colonization

S. aureus colonizes the skin and mucosal surfaces such as the nose and throat, as well as the axillae, vagina, and perineum; however, the preferred site of colonization seems to be the anterior nares (22–25). S. aureus binding to mucin is an important factor in the organism’s ability to colonize the nasopharyngeal mucosal surfaces (26). Approximately 30% of the population are transient carriers, and 20% are persistent carriers of S. aureus in the anterior nares (27). Moreover, there is a greater possibility of recovering S. aureus from other body sites among these carriers compared to non-carriers (1). In fact, self-infection in carriers of S. aureus has been reported as early as in the late 1950s. Williams et al. in 1959 noticed that infectious staphylococcal strains were normally the same strains that colonized the nares in the same individual (28). Additionally, Von Eiff et al. in 2001 reported that 82% of their sampled bacteremia patients displayed diseases caused by staphylococcal strains of endogenous origin (29). Strains obtained from the nares of these patients were identical to those in the blood up to 14 months later (28, 29).

Another target of S. aureus colonization, and subsequent infection, is the skin that represents the residence of multiple microorganisms including bacteria, viruses, fungi and
even mites (30–32). The microbiome that makes up the skin is more variable over time when compared to that of the gut and mouth (33). The skin microbiome can vary with topography, thickness, and frequency of cutaneous appendages such as sweat, sebaceous glands, and hair follicles (34). Sebum, for instance, is a lipid-rich antibacterial coating that lubricates and protects the skin. It is secreted from the sebaceous glands and differentiated keratinocytes in the stratum corneum of the skin. These sebaceous glands are associated with the hair follicles, and support anoxic conditions allowing for selection of facultative anaerobic microorganisms. Studies have shown that *Propionibacterium acnes*, for instance, can grow in sebaceous glands and secrete lipases that break down sebum lipids (35). Sebum triglycerides undergo enzymatic hydrolysis by *P. acnes* resulting in the release of free fatty acids on the skin (36, 37). Additionally, hormone production, life style choices such as occupation and antibiotic use, and the genetic profile of the host also contribute to the variability of the skin microbiome (38–40). Staphylococcal species are considered one of the most stable inhabitants of the skin (41). They have developed mechanisms that enable them to inhabit moist skin microenvironments, most likely by utilizing the urea component of sweat as a nitrogen source (42). Breaching cutaneous immunity on the skin provides an opportunity for pathobionts, like *S. aureus*, to establish infections. The introduction of foreign objects, such as indwelling medical devices, also provide a niche for staphylococcal colonization. Moreover, members of the resident skin microbiota might contribute to cutaneous immunity, as was reported by Iwase *et al.* in which a subset of *Staphylococcus epidermidis* strains evolved to produce an endopeptidase protein that cooperatively works with the host's β-defensin 2 to impede *S. aureus* colonization and biofilm formation (43).

1.4 *Staphylococcus aureus* pathogenesis and clinical manifestation

Although *S. aureus* is considered a normal constituent of the human microbiota, it is also a highly successful opportunistic pathogen (1). Upon breach of cutaneous immunity, *S. aureus* excels at epithelial adhesion, invasion, and subsequently immune evasion to establish a successful infection. To accomplish pathogenesis, *S. aureus* employs a collection of virulence determinants that can be divided into three categories: secreted
enzymes; adhesion and immune evasion factors; and toxins. Secreted enzymes that are associated with *S. aureus* adhesion and invasion include: glycerol ester hydrolases that are involved in degradation of triacylgercylols; phosphatidylinositol-specific lipase (PtdIns-phospholipase C); and enolases that mediate binding to laminin (44–49). Adhesion factors include a plethora of microbial surface proteins known collectively as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (50). The acronym was first used in 1994 to describe microbial adhesins, such as fibronectin-binding proteins A and B, collagen-binding adhesin, and *S. aureus* surface protein G/S, that span the staphylococcal wall, and have exposed domains which recognize host proteins such as fibronectin, fibrinogen, laminin, collagen, and heparin associated polysaccharides with a high degree of specificity (50–59). Subsequently, *S. aureus* is able to evade host immune defenses with factors such as staphylococcal protein A which binds immunoglobulins to block phagocytosis, and toxins such as δ toxin which is involved in neutrophil lysis and the lethal α toxin (60–70). A brief summary of *S. aureus* virulence determinants and their functions is listed in Table 1.1.
### Table 1.1 Summary of *S. aureus* virulence determinants.

<table>
<thead>
<tr>
<th>Category</th>
<th>Determinant</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secreted enzymes</td>
<td>SspA (serine protease)</td>
<td>Cleaves fibronectin-binding protein as well as cleaves the heavy chains of all human immunoglobulin classes.</td>
<td>(71–74)</td>
</tr>
<tr>
<td></td>
<td>PtdIns-phospholipase C</td>
<td>Phosphatidylinositol-specific lipase activity that releases glycan-PtdIns-anchored cell surface proteins.</td>
<td>(45, 48)</td>
</tr>
<tr>
<td></td>
<td>Enolase</td>
<td>Mediates the binding to laminin.</td>
<td>(45, 49)</td>
</tr>
<tr>
<td></td>
<td>glycerol ester hydrolase</td>
<td>Involved in degradation of triacylglycerols, and impede human granulocyte function.</td>
<td>(44, 45)</td>
</tr>
<tr>
<td>Adhesion factors</td>
<td>Fibronectin-binding proteins A and B</td>
<td>Bind fibronectin, fibrinogen and elastin.</td>
<td>(51–54)</td>
</tr>
<tr>
<td></td>
<td><em>S. aureus</em> surface protein G and S</td>
<td>Binds to the extracellular matrix.</td>
<td>(58, 59)</td>
</tr>
<tr>
<td></td>
<td>Collagen-binding adhesin</td>
<td>Binds collagen I and IV.</td>
<td>(55–57)</td>
</tr>
<tr>
<td></td>
<td>Elastin-binding protein</td>
<td>Binds to elastin.</td>
<td>(75, 76)</td>
</tr>
<tr>
<td></td>
<td>von Willebrand factor binding protein</td>
<td>Binds von Willebrand factor and fibrinogen. Also binds and</td>
<td>(47)</td>
</tr>
<tr>
<td>Adhesion and immune evasion factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Extracellular adherence protein</td>
<td>Binds to extracellular matrix. Also displays immunomodulatory properties.</td>
<td></td>
<td>(77–81)</td>
</tr>
<tr>
<td>Extracellular matrix protein-binding protein</td>
<td>Binds to the extracellular matrix.</td>
<td></td>
<td>(82)</td>
</tr>
<tr>
<td>Bone sialoprotein-binding protein</td>
<td>Binds bone sialoprotein as well as fibrinogen.</td>
<td></td>
<td>(83–85)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Immune evasion factors</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylokinase</td>
<td>Involved in plasminogen activation and defensin inactivation. Displays anti-opsonic and fibrinolytic properties.</td>
<td></td>
<td>(93–96)</td>
</tr>
<tr>
<td>Protein A</td>
<td>Binds immunoglobulins, tumor-necrosis factor-alpha receptor 1 and von Willebrand factor.</td>
<td></td>
<td>(61–63)</td>
</tr>
<tr>
<td>Staphylococcal complement inhibitor</td>
<td>Acts on C3 convertases to inhibit complement</td>
<td></td>
<td>(97)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------------------------------------------------------------</td>
<td>------------------------------------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>Chemotaxis inhibitory</td>
<td>Binds to C5a and blocks chemotaxis</td>
<td></td>
<td>(98, 99)</td>
</tr>
<tr>
<td>protein</td>
<td>Binds fibrinogen as well as complement</td>
<td>Binds fibrinogen as well as complement</td>
<td></td>
</tr>
<tr>
<td>Extracellular fibrinogen-</td>
<td>C3. Recruits plasmin to degrade C3 and C3b. Inactivates</td>
<td>C3. Recruits plasmin to degrade C3 and C3b. Inactivates</td>
<td>(100–103)</td>
</tr>
<tr>
<td>binding protein</td>
<td>complement.</td>
<td>complement.</td>
<td></td>
</tr>
<tr>
<td>Capsular polysaccharides</td>
<td>Impede phagocytosis</td>
<td>Modifies membrane phosphatidylglycerol with 1-lysine. Mediates</td>
<td>(104–106)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>resistance to cationic antimicrobial peptides and defensins</td>
<td></td>
</tr>
<tr>
<td>Multiple peptide</td>
<td>Modifies membrane phosphatidylglycerol with 1-lysine. Mediates</td>
<td>C3b. Inactivates complement.</td>
<td>(107, 108)</td>
</tr>
<tr>
<td></td>
<td>as well as evasion of</td>
<td>as well as evasion of</td>
<td></td>
</tr>
<tr>
<td>Antimicrobial peptide</td>
<td>Two component histidine kinase and response regulator system</td>
<td>Two component histidine kinase and response regulator system</td>
<td>(109, 110)</td>
</tr>
<tr>
<td>sensing system</td>
<td>that is involved in antimicrobial</td>
<td>that is involved in antimicrobial</td>
<td></td>
</tr>
<tr>
<td></td>
<td>peptides resistance</td>
<td>peptides resistance</td>
<td></td>
</tr>
<tr>
<td>Toxins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β hemolysin</td>
<td>Cytotoxic action.</td>
<td>Cytotoxic action.</td>
<td>(111, 112)</td>
</tr>
<tr>
<td>Toxins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>δ toxin</td>
<td>Cytotoxicity and membrane-damaging properties as well as</td>
<td>Cytotoxicity and membrane-damaging properties as well as</td>
<td>(64, 65)</td>
</tr>
<tr>
<td></td>
<td>induction of tumor</td>
<td>induction of tumor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>necrosis factor alpha</td>
<td>necrosis factor alpha</td>
<td></td>
</tr>
<tr>
<td>Toxin Type</td>
<td>Activity in Macrophages/Neutrophils</td>
<td>Reference(s)</td>
<td></td>
</tr>
<tr>
<td>------------------------------------</td>
<td>--------------------------------------------------------------------------------------------------</td>
<td>--------------------</td>
<td></td>
</tr>
<tr>
<td>γ toxin</td>
<td>Hemolytic activity</td>
<td>(113, 114)</td>
<td></td>
</tr>
<tr>
<td>Enterotoxins</td>
<td>Superantigen with immunomodulatory properties via potent T cell activation.</td>
<td>(115, 116)</td>
<td></td>
</tr>
<tr>
<td>Toxic shock syndrome toxin 1</td>
<td>Superantigen with immunomodulatory properties via potent T cell activation.</td>
<td>(115, 117–119)</td>
<td></td>
</tr>
<tr>
<td>Leukocidins A, B, D, E and M.</td>
<td>LukA/B have cytolytic activity towards macrophages and neutrophils. Luk D/E/M have leukocidal activity.</td>
<td>(120–123)</td>
<td></td>
</tr>
<tr>
<td>Panton-Valentine leukocidin</td>
<td>Pore-forming toxin with cytolytic activity towards macrophages and neutrophils.</td>
<td>(124–127)</td>
<td></td>
</tr>
</tbody>
</table>
S. aureus can cause mild skin and soft tissue infections as well as life-threatening infections such as infective endocarditis, osteomyelitis, and sepsis. In the early 1990s, these infections were primarily nosocomial, and spread rapidly in healthcare settings. In one report, a single MRSA strain spread in two hospitals in British Colombia and Manitoba within 6 weeks from the strain’s introduction from the Punjab (128). First reports of CA-MRSA infections were documented in the United States in 1997, and since then CA-MRSA strains have proven to be hyper-virulent especially those belonging to the USA300 lineage (129). USA300 is particularly hyper-virulent for multiple reasons: first, it carries a smaller staphylococcal cassette chromosome (SCCmec) than that possessed by hospital-acquired strains, and thus has a relatively lower fitness cost (130); second, it is notorious for causing destructive infections such as necrotizing pneumonia due, in part, to the production of Panton-Valentine leucocidin (PVL) that causes leukocyte destruction and tissue necrosis (16, 131); third, it has acquired the arginine catabolic mobile element (ACME) from the skin commensal S. epidermidis which encodes determinants of resistance and virulence that enhance pathogenesis (132).

1.5 Genetic regulation of Staphylococcus aureus virulence

In addition to the cell wall associated factors, enzymes, and toxins mentioned above, S. aureus virulence is regulated extensively at the transcriptional level. This regulation is rather complex and involves a combination of multiple sensory and regulatory systems. The principal regulatory system in S. aureus is the accessory gene regulator, agr. The agr locus contains four genes, agrBDCA, and RNAIII which is the effector molecule of this global regulatory locus. The gene agrB encodes a membrane-associated protease that alters the agrD-encoded prepropeptide. This alteration results in the production of the small peptide molecule, which is the signal for membrane associated sensor kinase encoded by agrC. The recognition of this signal then results in AgrC activating the response regulator that is encoded by agrA, and this in turn upregulates RNAIII expression. (133–135). RNAIII governs the regulation of an array of virulence determinants, both at the transcriptional and translational levels. Additionally, there are other two-component regulatory systems and global transcriptional regulators that work with, and independent of, the agr system to govern the pleiotropic regulation of
staphylococcal virulence. A brief summary of those regulatory systems and the effect they exert on major virulence determinants can be found in Table 1.2
Table 1.2 Major regulators of virulence determinants in *S. aureus*.

<table>
<thead>
<tr>
<th>The regulatory system</th>
<th>Genes regulated</th>
<th>The type of regulation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accessory gene regulator (<em>agr</em>)</td>
<td>Protein A</td>
<td>-</td>
<td>(136)</td>
</tr>
<tr>
<td></td>
<td>Fibronectin-binding protein</td>
<td>-</td>
<td>(137)</td>
</tr>
<tr>
<td></td>
<td>PVL</td>
<td>+</td>
<td>(138)</td>
</tr>
<tr>
<td></td>
<td>SspA (V8 serine protease)</td>
<td>+</td>
<td>(139)</td>
</tr>
<tr>
<td></td>
<td>Leukotoxin LukE/D</td>
<td>+</td>
<td>(138)</td>
</tr>
<tr>
<td></td>
<td>α-hemolysin</td>
<td>+</td>
<td>(140)</td>
</tr>
<tr>
<td></td>
<td>β-hemolysin</td>
<td>+</td>
<td>(141)</td>
</tr>
<tr>
<td></td>
<td>δ-hemolysin</td>
<td>+</td>
<td>(141)</td>
</tr>
<tr>
<td></td>
<td><em>agr</em></td>
<td>+</td>
<td>(142)</td>
</tr>
<tr>
<td></td>
<td>Protein A</td>
<td>-</td>
<td>(143)</td>
</tr>
<tr>
<td></td>
<td>Fibronectin-binding protein</td>
<td>+</td>
<td>(137)</td>
</tr>
<tr>
<td></td>
<td>PVL</td>
<td>+</td>
<td>(138)</td>
</tr>
<tr>
<td></td>
<td>SspA</td>
<td>-</td>
<td>(139)</td>
</tr>
<tr>
<td></td>
<td>Leukotoxin LukE/D</td>
<td>+</td>
<td>(138)</td>
</tr>
<tr>
<td></td>
<td>α-hemolysin</td>
<td>+</td>
<td>(144)</td>
</tr>
<tr>
<td>S. aureus exoprotein (sae)</td>
<td>δ-hemolysin</td>
<td>+</td>
<td>(143)</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------</td>
<td>---</td>
<td>-------</td>
</tr>
<tr>
<td></td>
<td>Toxic shock syndrome toxin 1</td>
<td>-</td>
<td>(145)</td>
</tr>
<tr>
<td></td>
<td>agr</td>
<td>-</td>
<td>(146)</td>
</tr>
<tr>
<td></td>
<td>sarA</td>
<td>-</td>
<td>(146)</td>
</tr>
<tr>
<td></td>
<td>Protein A</td>
<td>+</td>
<td>(147)</td>
</tr>
<tr>
<td></td>
<td>Fibronectin-binding protein</td>
<td>+</td>
<td>(147)</td>
</tr>
<tr>
<td></td>
<td>SspA</td>
<td>+</td>
<td>(148)</td>
</tr>
<tr>
<td></td>
<td>α-hemolysin</td>
<td>+</td>
<td>(149)</td>
</tr>
<tr>
<td></td>
<td>β-hemolysin</td>
<td>+</td>
<td>(149)</td>
</tr>
<tr>
<td></td>
<td>Coagulase</td>
<td>+</td>
<td>(149)</td>
</tr>
<tr>
<td></td>
<td>Toxic shock syndrome toxin 1</td>
<td>+</td>
<td>(145)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Repressor of toxin (rot)</th>
<th>Protein A</th>
<th>+</th>
<th>(150)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-hemolysin</td>
<td>-</td>
<td>(151)</td>
</tr>
</tbody>
</table>
1.6 Antimicrobial Resistance mechanisms of 
*Staphylococcus aureus*

There are two mechanisms by which *S. aureus* acquires resistance to antimicrobials: horizontal transfer of an existing resistance gene, and through intrinsic mutations. The first evidence of resistance in *S. aureus* was to penicillin, which was mediated by β-lactamase encoded by the gene *blaZ*. Upon antibiotic exposure, a transmembrane signal transducer, BlaR1, undergoes proteolytic cleavage to inactivate a transcriptional repressor BlaI, and this de-repression permits expression of *blaZ*, encoding a β-lactamase. This enzyme hydrolyzes the β-lactam ring of the antibiotics rendering them ineffective (152, 153). The horizontal transfer of the *mec* gene accounted for the emergence of methicillin resistance among *S. aureus* strains. The *mecA* encodes the membrane-bound enzyme, penicillin-binding protein 2a (PBP2a). Typical PBPs catalyze the transpeptidation reaction required for peptidoglycan cross-linkage, and PBP2a exhibits low affinity to all agents of the β-lactam class of antibiotics. The expression of *mecA* is regulated in a similar fashion to that of *blaZ*, via the combined action of a sensor-transducer protein and a transcriptional repressor (MecR1 and MecI, respectively) (154–157). Resistance to quinolones, on the other hand, is an example of a resistance mechanism mediated by intrinsic mutation. Fluoroquinolones inhibit DNA gyrase and topoisomerase IV, which together mitigate DNA supercoiling. Mutations in the conserved quinolone resistance-determining regions reduce quinolone affinity and are associated with resistance in clinical isolates (158–160). Two resistance mechanisms to the glycopeptide antibiotic, vancomycin, have been proposed. The first mechanism is chromosomally mediated, attributed to mutations that increase synthesis of peptidoglycan and cell wall precursor, thus preventing the antibiotic from binding to its target (161–164). The second mechanism is mediated by acquisition of an enterococcal operon that is responsible for changing the terminal peptide of the cell wall precursor, thus reducing affinity for vancomycin (161–164).
1.7 *Staphylococcus aureus* envelope architecture

1.7.1 Cell envelope composition

Gram-positive bacteria are surrounded by a cell wall, composed of layers of peptidoglycan with attached accessory proteins and teichoic acids, as well as a cytoplasmic membrane. Teichoic acids can be either in the form of wall teichoic acids or lipoteichoic acids. Wall teichoic acids are anionic polymers of teichoic acids that are covalently anchored to the peptidoglycan layer of the cell envelope, whereas the lipoteichoic acids are those embedded into the cytoplasmic membrane via a lipid moiety. Wall teichoic acids are comprised of repetitive units of ribitol phosphate or glycerol phosphate, and are linked to the N-acetylmuramic acid (MurNAc) of the peptidoglycan through a disaccharide of N-acetylglucosamine (GlcNAc)-1-P and N-acetylmannosamine (ManNAc) which is followed by glycerol phosphate units (165, 166). In contrast, lipoteichoic acids are comprised of repetitive units of glycerol phosphate with a D-alanyl ester or an α-GlcNAc on the second hydroxyl group of the glycerol, and are linked by a diglucosyl diacylglycerol anchor to the outer leaflet of cytoplasmic membrane (167). *S. aureus* utilizes the presence of wall polymers to defend itself from environmental stressors. Studies have shown that lipoteichoic acids surface-anchoring properties play an integral role in invading the microvascular endothelial cells of the brain. *S. aureus* mutants deficient in lipoteichoic acid membrane anchoring were found to be impaired in their capability to penetrate the blood-brain barrier thus reducing the chance of staphylococcal central nervous system disease (168). These wall polymers have also been linked to resistance to cationic antimicrobial peptides and cationic antibiotics (169, 170).

Gram-positive bacteria are also surrounded by a single cytoplasmic membrane. The barrier function of the membrane is mediated by its phospholipid bilayer that surrounds and protects the cytoplasm. There are three major polar phospholipids in *S. aureus* membranes: phosphatidylglycerol; lysyl-phosphatidylglycerol; and cardiolipin, which is the major phospholipid during stationary phase (171). Phospholipid modifications play a vital role in resistance to host defenses and antimicrobial substances. Failure to modify phosphatidylglycerol with L-lysine, to form lysyl-phosphatidylglycerol, reduces the positive charge of the cell envelope, rendering *S. aureus* more susceptible to cationic
antimicrobial peptides (107). Furthermore, membrane phospholipids composition must be altered to accommodate environmental stressors such as fluctuations in temperature and pH, and exogenous antimicrobials.

1.7.2 Membrane homeostasis

Fatty acids play important roles in microorganisms, both as an integral component of cellular membranes, as well as a source of energy. While the biosynthetic pathways for these fatty acids are rather complex and energetically expensive, a stable membrane composition must be maintained. The Gram-negative *Escherichia coli* utilizes the combination of transcriptional activators and repressors, belonging to the TetR and GntR families of transcriptional regulators, to control genes involved in fatty acid metabolism in order to maintain membrane homeostasis (172, 173). This homeostasis especially must be maintained upon exposure to exogenous fatty acids that could interfere with membrane fluidity. Host-derived fatty acids are either utilized as a precursor in the membrane phospholipid biosynthetic pathway or degraded via the β-oxidation pathway to be utilized as an energy source for the bacteria. Fatty acid biosynthesis is regulated via the TetR family regulator, FabR, and fatty acid degradation is regulated via the GntR family regulator, FadR (172, 174). FadR is a repressor of the β-oxidation pathway (*fad* regulon) and an activator for the *fabA* and *fabB* genes, which in turn are under the regulation of the repressor FabR, and acyl-CoA thioesters are the ligand that modulates binding of FadR and FabR to their operator DNA (173–175). Therefore, membrane lipid homeostasis in *E. coli* is a product of a transcriptional regulator with dual functionality that contributes to both fatty acid synthesis and degradation. An overview of the regulation of lipid homeostasis in *E. coli* can be seen in Figure 1.1
In the absence of acyl-CoA

Repression of \( fad \) genes involved in fatty acid degradation (\( \beta \)-oxidation) pathway

Activation of \( fab \) genes involved in fatty acid biosynthesis pathway

In the presence of acyl-CoA

Activation of \( fad \) genes involved in fatty acid degradation (\( \beta \)-oxidation) pathway

Repression of \( fab \) genes involved in fatty acid biosynthesis pathway

Figure 1.1 Regulation of lipid homeostasis in \( E. \ coli \). Exogenous fatty acids are converted to acyl-CoAs after entry into the cell. In the absence of acyl-CoA, the transcriptional regulator FadR acts as a repressor of the \( fad \) regulon to repress fatty acid degradation, and as an activator of \( fabA \) and \( fabB \) to activate fatty acids biosynthesis. The genes \( fabA \) and \( fabB \) are also under the control of the transcriptional repressor FabR. In the presence of acyl-CoA, FadR dissociates from its operator DNA, resulting in the expression of \( fad \) genes to activate fatty acid degradation, as well as repression of the \( fab \) genes to repress fatty acid synthesis (176, 177).
Moreover, optimal membrane fluidity is governed by the fatty acid composition of phospholipids; both saturated and unsaturated. Saturated fatty acids are produced to increase membrane rigidity, whereas unsaturated fatty acids are produced to increase membrane fluidity, as is the case when the bacteria are exposed to higher and lower growth temperatures, respectively (178). For instance, *Pseudomonas aeruginosa* employs two desaturase systems, in addition to the Fab pathway, to regulate fatty acid biosynthesis to maintain membrane homeostasis. The first system is DesA which modifies existing membrane phospholipids to accommodate fluctuations in fluidity. The second desaturase system is DesB which alters saturated fatty acids of exogenous source, such as those lipids in the pulmonary surfactant encountered by *P. aeruginosa*, to produce unsaturated fatty acids to maintain optimal membrane fluidity. DesB is regulated at the transcriptional level by DesT which is a regulator that senses environmental fatty acids, and differentiates between saturated and unsaturated acyl-CoA substrates. In the presence of unsaturated acyl-CoA, DesT binds to an operator DNA located in the promoter region of desCB, to repress transcription of these genes which are necessary for production of unsaturated fatty acids. Conversely, the presence of saturated acyl-CoA causes the release of DesT from the operator DNA and the subsequent expression of desB, which in turn introduces double bonds into the acyl-CoA products of exogenous fatty acids and restores optimal membrane fluidity (179–181).

Comparatively, transcriptional regulation of membrane lipid homeostasis in Gram-positive bacteria is less well understood. The most detailed studies have been conducted in *Bacillus subtilis*, where a two-component desaturase system encoded by desK and desR regulates the membrane-bound acyl desaturase, DesA. This desaturase system senses temperature fluctuations and since *B. subtilis* is incapable of de novo synthesis of unsaturated fatty acids, the bacterium employs DesA to desaturate existing phospholipids in order to maintain membrane fluidity (182, 183). Additionally, *B. subtilis* also employs a transcriptional regulator, FadR, which controls genes involved in fatty acid degradation. Unlike FadR of *E. coli*, which is a GntR regulator, FadR of *B. subtilis* is a TetR family regulator. Similar to *E. coli*, *B. subtilis* FadR is a repressor of genes involved in fatty acid degradation, and acyl-CoA is the ligand that modulates binding of FadR to its operator
DNA. In the presence of exogenous fatty acids, the promoters repressed by FadR are de-repressed and fatty acids are degraded to be utilized as a carbon source (184)

Furthermore, *B. subtilis* employs transcriptional regulation to sense the status of the intracellular pool of fatty acids to maintain lipid homeostasis and optimal membrane fluidity. The repressor FapR regulates the expression of genes involved in fatty acid and phospholipid synthesis in response to the cellular levels of malonyl-CoA. Elevated levels of malonyl-CoA are indicative of diminished fatty acid and phospholipid synthesis which in turn results in the de-repression of FapR-mediated genes that are involved in lipid biosynthetic machinery (185, 186). FapR is the first global transcriptional regulator that is highly conserved among Gram-positive bacteria (185, 187). FapR was first characterized and purified from *B. subtilis*, and was subsequently found to be conserved in other organisms such as *S. aureus* and *Listeria monocytogenes*. Similar to FapR in *B. subtilis*, staphylococcal FapR senses malonyl-CoA to monitor lipid synthesis in a feed-forward mechanism to maintain membrane homeostasis (185, 188). This feed-forward mechanism involves recognition of an upstream biosynthetic product to regulate the transcription of the genes involved in the lipid biosynthetic machinery.
A. **In the absence of acyl-CoA**

- FadR
- Repression of genes involved in fatty acid degradation

B. **In the presence of acyl-CoA**

- FadR
- De-repression of genes involved in fatty acid degradation

**B. Low cellular pool of malonyl-CoA**

- FapR
- Repression of *fap* genes involved in fatty acid and phospholipid biosynthesis pathway

**Elevated cellular pool of malonyl-CoA**

- FapR
- Relief of FapR mediated repression of genes involved in fatty acid and phospholipid biosynthesis pathway
**Figure 1.2 Regulation of lipid homeostasis in *B. subtilis*.** A. Exogenous fatty acids are converted to acyl-CoAs after entry into the cell. In the absence of acyl-CoA, the transcriptional regulator FadR acts as a repressor of the *fad* regulon to repress fatty acid degradation. In the presence of acyl-CoA, FadR dissociates from its operator DNA, resulting in the activation of fatty acid degradation pathway to utilize the exogenous fatty acids as a carbon source. B. FapR is responsive to the cellular status of fatty acid biosynthesis. Malonyl-CoA is the ligand modulating FapR binding to its operator DNA. In low levels of malonyl-CoA, fatty acid biosynthesis genes are repressed. In the presence of malonyl-CoA, FapR dissociates from its operator DNA, resulting in the expression of *fap* genes to activate fatty acid and phospholipid synthesis.
1.8 Fatty acid machinery of *Staphylococcus aureus*

1.8.1 Phospholipid composition

There are three major phospholipids in *S. aureus* membranes: phosphatidylglycerol; lysyl- phosphatidylglycerol; and cardiolipin (171). These phospholipids play a crucial role in preserving membrane biophysical properties, and particularly, the fatty acid composition of phospholipids is the key determinant of membrane fluidity (189). Unlike *B. subtilis* that possesses a gene encoding a membrane phospholipid desaturase for the synthesis of unsaturated fatty acids, *S. aureus* does not possess such desaturase and thus is incapable of producing these fatty acids (182). Instead, a combination of branched- and straight-chain fatty acids are present in staphylococcal membranes. Branched-chain fatty acids are the predominant fatty acids in *S. aureus* phospholipids, comprising 55-65% of the total fatty acids (189, 190). Branched-chain fatty acids are synthesized from branched-chain amino acids; these include leucine- and valine-derived (iso) fatty acids, and isoleucine-derived (anteiso) fatty acids, with the latter in particular promoting increased membrane fluidity (189, 191).

1.8.2 Phospholipid synthesis

The biosynthetic pathway of staphylococcal branched-chain fatty acids starts when the amino acids leucine, isoleucine and valine undergo a transamination reaction mediated by the branched-chain amino acid transaminase BAT. The resulting branched-chain α-keto acid then undergoes a decarboxylation reaction mediated by α-keto acid dehydrogenase (BKD), resulting in the formation of branched-chain acyl coenzyme A derivatives which are the precursor for fatty acids biosynthesis. The branched-chain acyl coenzyme A derivatives, in combination with malonyl-ACP, are then utilized by the β-ketoacyl-ACP synthase III (FabH) to generate β-ketoacyl-ACP. This in turn undergoes a reduction reaction mediated by FabG reductase, a dehydration reaction mediated by FabZ and a reduction reaction mediated by FabI, resulting in the formation of saturated acyl-ACP. The enzyme FabF catalyzes the condensation reaction permitting further product elongation. The resulting long chain acyl-ACP is then used in the generation of the phospholipid precursor, phosphatidic acid (189, 192–196).
The peripheral membrane protein PlsX is responsible for transferring the acyl group from Acyl-ACP to an inorganic phosphate, forming an acylphosphate product, which can then be used by an integral plasma membrane protein, PlsY. This interaction between PlsY and the acylphosphate product, in the presence of glycerol-3-phosphate, results in the formation of Acyl-G3P, which is then acylated by the integral membrane protein PlsC, forming phosphatidic acid that represents the key intermediate in membrane phospholipid formation. Phosphatidic acid is subsequently used, in the presence of cytidine triphosphate, to synthesize cytidine diphosphate diacylglycerol through the action of phosphatidate cytidylyltransferase. Phosphatidylglycerol-phosphate is then generated upon the replacement of cytidine monophosphate with glycerol phosphate. The dephosphorylation of phosphatidylglycerol-phosphate results in the formation of phosphatidylglycerol which is the core phospholipid in S. aureus (196–201). An overview of fatty acid and phospholipid biosynthesis in S. aureus can be seen in Figure 1.

In addition to the fatty acids produced by the fatty acid synthase machinery mentioned above, S. aureus is also capable of utilizing fatty acids of exogenous source. Upon entry into the cell by flipping across the membrane leaflet, exogenous fatty acids are processed by the fatty acid kinase machinery. This machinery includes a kinase domain protein, FakA, and a fatty acid binding protein FakB1 and FakB2 for binding saturated and unsaturated fatty acids, respectively. Exogenous fatty acids bind FakB, and after phosphorylation by FakA, are incorporated into the phospholipid directly, or indirectly after passing through an extension cycle by the FASII machinery (202). During the extension cycle, acyl chains are extended by two carbons via four enzymatic reactions. Extension is initiated by an elongation condensing enzyme, FabF, and the resulting β-ketoacyl-ACP is then reduced by the reductase, FabG. Subsequently, β-hydroxyacyl-ACP undergoes a dehydration reaction through the action of FabZ. The resulting trans-2-enoyl-ACP undergoes a reduction reaction that is catalyzed by the reductase FabI to complete the extension cycle (203–206). The peripheral membrane protein PlsX is responsible for transferring the acyl group from the extended fatty acid product to an inorganic phosphate, forming an acylphosphate product, which can then be used by an
integral plasma membrane protein, PlsY. This interaction between PlsY and the acylphosphate product results in the acylation of glycerol-3-phosphate forming the first intermediate in membrane phospholipid formation, known as lysophosphatidic acid. Lysophosphatidic acid is then acylated by the integral membrane protein PlsC, forming phosphatidic acid that represents the core intermediate in membrane phospholipid formation (197–200). An overview of exogenous fatty acid utilization in S. aureus can be seen in Figure 1.4
Leucine
Valine
Isoleucine

Branched-chain amino acid transaminase

Branched-chain β-keto acids

α-keto acid dehydrogenase

Branched-chain acyl coenzyme A derivatives

+ malonyl-ACP

FabH

β-ketoacyl-ACP

FabG
FabZ
FabI

Saturated acyl-ACP

FabF

Long chain acyl-ACP

PlsX
Acyl- PO$_4$

+ Glycerol 3-phosphate

PlsY
Acyl- G3P

PlsC

Phosphatidic acid

Cytidine diphosphate diacylglycerol

Phosphatidylglycerol phosphate

Phosphatidylglycerol
Figure 1.3 Overview of fatty acid and phospholipid biosynthesis in *S. aureus*.

Synthesis of staphylococcal branched-chain fatty acids starts when the amino acids leucine, isoleucine and valine undergo a transamination followed by a carboxylation reactions reaction, resulting in the formation of branched-chain acyl coenzyme A derivatives which are the precursor for fatty acids biosynthesis. The branched-chain acyl coenzyme A derivatives, in combination with malonyl-ACP, are then utilized by FabH to generate β-ketoacyl-ACP. This in turn undergoes a reduction reaction by FabG, a dehydration reaction by FabZ and a reduction reaction by FabI, resulting in the formation of saturated acyl-ACP. The enzyme FabF catalyzes the condensation reaction permitting further product elongation where the resulting acyl-ACP can undergo additional rounds of elongation by FabG, FabZ and FabI. For simplicity, the steps shown here are unidirectional. Subsequently, the resulting long chain acyl-ACP is then used in the generation of the phospholipid precursor, phosphatidic acid. The protein PIsX is responsible for transferring the acyl group from Acyl-ACP to an inorganic phosphate, forming an acylphosphate product, which can then be used by PIsY to form acyl-G3P. The latter in turn is acylated by PIsC, forming phosphatidic acid which is subsequently used, in the presence of cytidine triphosphate, to synthesize cytidine diphosphate diacylglycerol through the action of phosphatidate cytidylyltransferase. Phosphatidylglycerol-phosphate is then generated upon the replacement of cytidine monophosphate with glycerol phosphate, and subsequently dephosphorylated to form phosphatidylglycerol which is the core phospholipid in *S. aureus* membranes. Figure is adapted from Schiebel *et al*. and Kuhn *et al*. (193, 196).
Figure 1.4 Overview of exogenous fatty acid utilization by *S. aureus*. Upon entry into the cell, exogenous fatty acids (FA) are processed by the fatty acid kinase machinery; FA are bound by FakB and phosphorylated by FakA. Subsequently, phosphorylated fatty acids are either incorporated into the phospholipid directly (A), or indirectly after passing through an extension cycle by the FASII machinery (B). Figure is adapted from Parsons *et al.*, and Yao and Rock (202, 207).
1.8.3 Lipids as antimicrobials

The skin is the first line of defense against microbial infection. One of the host innate defense elements on the skin is antimicrobial long-chain unsaturated fatty acids. The main source of these fatty acids is the sebum secreted by the sebaceous glands and differentiating keratinocytes present in the stratum corneum of the epidermis (208). The sebum consists of squalene, wax monoesters, triglycerides, and small amounts of cholesterol and cholesterol esters (209). As the sebum’s constituents stream outwards through the hair follicle associated with the sebaceous glands, the triglycerides undergo enzymatic hydrolysis to produce unsaturated free fatty acids (uFFA) on the skin surface. The principal uFFA derived from sebaceous triglycerides is sapienic acid, which is a 16-carbon fatty acid with one degree of unsaturation that has potent bactericidal property against MRSA (208). Linoleic acid was identified as the major uFFA in human nasal secretion and is also present in S. aureus abscesses, a hallmark of S. aureus skin and soft tissue infections (210–212). Humans deficient in the production of uFFAs are more susceptible to S. aureus skin infections (213).

Although S. aureus possess the genes coding for enzymes involved in fatty acid degradation, studies have ruled out their involvement in exogenous fatty acid utilization (214). Alternatively, the only metabolic fate of exogenous uFFAs is through incorporation into the membrane phospholipid (194, 215). This incorporation is the rate limiting step such that when the levels of exogenous uFFAs exceeds that of the phospholipid biosynthetic machinery, uFFAs accumulate in the membrane and become detrimental (216). Although it has been long recognized that uFFAs have membrane damaging properties (217, 218), the exact mechanism by which these uFFAs exert their toxicity remained debatable. Several mechanisms have been proposed; these include uFFAs increasing the permeability of bacterial membranes due to their surfactant action, and disrupting the anisotropic nature of membranes by interfering with protons movement across the membrane and subsequently ATP synthesis (219, 220). Other reports have proposed that the toxic effects of uFFAs are mediated through a peroxidative process and through inhibiting fatty acid synthesis by acting as selective inhibitors of the enoyl –acyl carrier protein reductase (FabI) (221, 222). However, recent reports confirm
that exogenous uFFAs disrupt the cytoplasmic membrane integrity, resulting in dissolulion of the proton gradient across the membrane. This in turn leads to leakage of cellular metabolites and low-molecular weight proteins, and the eventual cessation of macromolecular biosynthetic pathways and loss of cellular viability (216).
Table 1.3 Summary of proposed mechanisms of uFFA toxicity.

<table>
<thead>
<tr>
<th>Target</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasmic membrane</td>
<td>Uncouple the electron transport chain</td>
<td>(223, 224)</td>
</tr>
<tr>
<td>Cytoplasmic membrane</td>
<td>Impede oxidative phosphorylation</td>
<td>(220)</td>
</tr>
<tr>
<td>Cytoplasmic membrane</td>
<td>Surfactant action that increases membrane permeability</td>
<td>(219)</td>
</tr>
<tr>
<td>Cytoplasmic membrane</td>
<td>Increase membrane fluidity leading to cell lysis</td>
<td>(218, 225)</td>
</tr>
<tr>
<td>Cytoplasmic membrane</td>
<td>Dissolution of the proton gradient across the membrane and subsequent release of low molecular-weight proteins</td>
<td>(216)</td>
</tr>
<tr>
<td>Fatty acid synthetic machinery</td>
<td>Enzymatic inhibition</td>
<td>(222)</td>
</tr>
<tr>
<td>Cellular viability</td>
<td>Oxidative stress</td>
<td>(221, 226)</td>
</tr>
</tbody>
</table>
Although exogenous uFFAs display antimicrobial properties, bacterial species are equipped with intrinsic mechanisms of resistance to these uFFAs. External structures such as the outer membrane and cell wall of Gram-negative and positive bacteria, respectively, mediate protection against uFFAs through several mechanisms that are briefly reviewed here. First, transcriptomics and proteomics studies show that upon exposure to exogenous uFFAs, *S. aureus* upregulates genes involved in carotenoid biosynthesis, as well as those in cellular energy metabolism, peptidoglycan, and cell wall biosynthetic pathways (227). Cell wall components play an integral role in *S. aureus* susceptibility to antimicrobial uFFAs, as wall structure, thickness and properties can impede the entry of these uFFAs. It has been proposed that the iron-regulated surface determinant, IsdA, is involved in resistance to uFFAs where this cell wall anchored protein modifies cellular hydrophobicity. The C domain of IsdA extends into the staphylococcal cell wall, altering its charge and hydrophobicity thus rendering the cells less susceptible to host antimicrobial peptides and fatty acids that rely on hydrophobic interactions to penetrate the cellular membrane and manifest their effects (227, 228). Other reports suggest that *S. aureus* counteracts the uFFA-mediated increase in membrane fluidity by upregulating the production of staphyloxanthin, leading to a direct correlation between membrane stability, cellular pigmentation, and uFFAs-induced cellular killing (218, 227, 229). The production of staphyloxanthin is regulated by the sigma B (σ^B^) regulon as a general stress response to the presence of uFFAs (227). Additionally, it has been proposed that cell wall teichoic acids confer resistance to uFFAs, by acting as a filter to restrict their passage across the cell wall, in a structure-specific manner, and cells lacking teichoic acids exhibit enhanced leakage of intracellular content (216, 230). Moreover, the fatty acid modifying enzyme (FAME), responsible for fatty acid esterification, has also been linked to uFFA tolerance. FAME can esterify bactericidal fatty acids that are present in staphylococcal abscesses to various alcohols, thus enabling bacterial survival within tissues (227, 231). As is the case with the majority of antimicrobial resistance, it can be anticipated that resistance to uFFAs should also be mediated through efflux pumps. *Neisseria gonorrhoea*, for instance, exhibits an efflux system dedicated to extrusion of hydrophobic antimicrobials such as free fatty acids and bile salts (232, 233). Similarly, there are some reports of efflux pumps contributing to *S.*
*S. aureus* resistance to skin antimicrobials such as fatty acids and polyamines. Tet38 is a multidrug efflux transporter that confers resistance to tetracycline as well as palmitoleic acid, one of the main uFFA in human nasal secretion (221, 234). Tet38 is implicated in *S. aureus* survival and colonization on the skin due to its ability to transport antimicrobial free fatty acids thus conferring resistance to them (234). However, this efflux pump is not specific just for uFFAs, as it contributes to tetracycline resistance as well, and therefore a specific fatty acid efflux pump is yet to be identified in *S. aureus.*
Table 1.4 Summary of uFFA resistance mechanisms proposed to date.

<table>
<thead>
<tr>
<th>Target</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell wall physiology</td>
<td>Decrease cellular hydrophobicity and thus prevent uFFA penetration</td>
<td>(228)</td>
</tr>
<tr>
<td>Membrane stability</td>
<td>Increase staphyloxanthin production to counteract the uFFA-mediated increase in membrane fluidity</td>
<td>(218, 227, 229)</td>
</tr>
<tr>
<td>Wall teichoic acids</td>
<td>Prevent binding of uFFAs</td>
<td>(216, 230)</td>
</tr>
<tr>
<td>uFFA transport</td>
<td>Tet38-mediated efflux of uFFAs outside the cell</td>
<td>(234)</td>
</tr>
<tr>
<td>uFFA processing</td>
<td>FAME- medicated esterification of uFFAs to various alcohols</td>
<td>(227, 231)</td>
</tr>
</tbody>
</table>
1.9 Bacterial efflux pumps

One of the greatest innovations in modern medicine is the introduction of antibiotics in clinical practices to treat the otherwise life-threatening infectious diseases. However, bacterial pathogens were able to circumvent this progress by developing resistance mechanisms leading to infectious diseases that are once again, a major problem in the hospital and community settings. The World Health Organization continuously urges for the development of new bacterial therapeutic targets to combat the life-threatening multidrug resistance (235, 236).

The efficacy of antimicrobial compounds can be abrogated by a number of mechanisms including: reduced uptake; enzymatic inactivation; target site modification; and efflux from the cell (237, 238). There are five primary families of multidrug efflux pumps: resistance nodulation cell division (RND); major facilitator superfamily (MFS); ATP binding cassette (ABC) family; multidrug and toxic compound extrusion (MATE) family; and small multidrug resistance (SMR) family. In addition to their notable functions in multidrug resistance, the RND efflux pumps have been extensively studied for their roles in fatty acid efflux (239, 240). The most well characterized member of the RND family of multidrug efflux pumps is the AcrB pump of *E. coli*. AcrB uses proton motive force to extrude hydrophobic substances such as antibiotics, bile salts, and a range of cellular metabolites. This tripartite efflux system in *E. coli* is composed of the trimeric AcrB that spans the inner membrane, TolC which is an outer membrane channel, and the periplasmic adaptor protein AcrA (241–243). Another RND family membrane protein that has a role in lipid efflux is the mycobacterium membrane protein large (MMPL) group of proteins in *Mycobacterium tuberculosis*. MMPLs are co-localized with lipid-metabolizing enzymes, and are involved in the transport of methyl-branched lipids (244). MMPL-mediated lipid pathways have been implicated in bacterial intracellular persistence, and four of the MMPL proteins appear to be indispensable for *M. tuberculosis* in fully maintaining virulence in mice (244).
1.10 TetR family of transcriptional regulators and their biological functions

Transcriptional regulators are the key to most microbial adaptation strategies. These regulators normally contain a DNA- and ligand-binding domain that can be used to sense environmental signals and elicit a downstream response at the transcriptional level. There are many families of transcriptional regulators that have been grouped based on structural and functional similarities, such as the helix-turn-helix motif, which is the most common DNA-binding motif among prokaryotes (245–247). Members of these families have either an activation or a repression function, with a few families that display unconventional dual roles in activation and repression. Members of the LysR, IcIR, MarR, and Crp families of transcriptional regulators have displayed dual roles (248–251). Recently, there have been few reports of members of the TetR family of transcriptional regulators that display dual functionality (252, 253).

TetR family regulators (TFRs) have been well documented for their roles in environmental adaptation and antibiotic resistance (254). There are more than 2500 TFRs with functions ranging from multidrug resistance, biosynthesis of antibiotics, and pathogenicity in both Gram-positive and Gram-negative bacteria (255). TFRs are named after the well-characterized TetR, a repressor that controls expression of a membrane-associated protein, TetA, that exports tetracycline out of the bacterial cell. \textit{tetR} and \textit{tetA} are divergently transcribed, and the \textit{tetR} gene product controls expression of both \textit{tetA} and \textit{tetR}. When tetracycline is present and associated with Mg$^{2+}$, it binds to the TetR protein leading to a conformational change that renders TetR unable to bind to the DNA. As a result, \textit{tetR} and \textit{tetA}, both of which are repressed by TetR, are expressed (256–260).

A well-documented staphylococcal TFR is QacR, which is a repressor regulating the expression of QacA, a multidrug efflux pump. Lyon \textit{et al.} in 1984 showed that the \textit{S. aureus} plasmid pSK1 encodes resistance to quaternary ammonium antiseptic compounds. This resistance is mediated by energy-dependent efflux encoded by the \textit{qacA} gene that belongs to a regulatory circuit similar to that of the efflux-mediated resistance to tetracycline. This plasmid-encoded system also contained a transcriptional regulator with sequence similarity to the helix-turn-helix motif of the transcriptional repressor that
governs tetracycline-resistance in Gram-negative bacteria (261–264). By 1998, it was confirmed that QacA contains 14 transmembrane domains and that it belongs to the major facilitator superfamily of transporters, and that QacR is the repressor regulating the expression of this transporter (265, 266). QacR functions as a homodimer with an N-terminal DNA-binding domain recognizing an operator sequence located downstream from the divergent qacA promoter (266–268). This binding is considered unorthodox for a repressor as it may not physically block the binding RNA polymerase directly, but rather it hinders the transition of the transcription machinery. The regulator of intercellular adhesion (ica) operon of S. epidermidis is a TFR that is responsible for regulating the synthesis of polysaccharide intercellular adhesin (PIA) and, subsequently, biofilm formation (269). Two IcaR homodimers bind cooperatively to an operator site located upstream of the start codon of icaA to repress its transcription (270, 271). Upon ligand binding of streptomycin and gentamicin, IcaR dimers undergo a conformational change in the DNA binding domain, and the subsequent transcription of the divergent icaADBC operon (272). Cramton et al in 1999 showed that the ica locus is not only present in S. aureus, but is also implicated for biofilm formation (273); however, in S. aureus, the teicoplanin-associated locus regulator (TcaR), a member of the MarR family of transcriptional regulators, is also a repressor of the ica operon (272).

Other examples of TFRs include: the AmeR of Agrobacterium tumefaciens which is a regulator of an RND- efflux system; AmrR of Pseudomonas aeruginosa which is a regulator of an efflux pump involved in aminoglycoside resistance; and BpeR of Burkholderia pseudomallei which is a repressor of an RND pump involved in gentamycin, streptomycin, and erythromycin resistance (274–276). Similarly, resistance to the anti-tuberculosis drug ethionamide in M. tuberculosis is mediated by the TetR regulator EthR, and resistance to hydrophobic antimicrobial agents that target N. gonorrhoea is mediated by MtrR (233, 277–279). TFRs are also involved in regulation of virulence genes, such as HapR, which is a density-dependent regulator of virulence in Vibrio cholera (280, 281).

Additionally, several TFRs have a role in regulation of fatty acid efflux. AcrR is a repressor of the multidrug efflux pump AcrAB in E. coli; a prototypic member of the
RND family of efflux pumps. AcrR interacts with the *acr* promoter located in the intergenic segment between *acrR* and *acrAB*. This interaction between AcrR and the promoter region is responsive to accumulation of cellular metabolites and bile salts as well as global stress signals. In the presence of inducing ligand, AcrR is de-repressed and functions in combination with global transcriptional activators to enable efflux pump expression (282–285). Other TFRs that have roles in lipid efflux include: CmeR from *Campylobacter jejuni* and MtrR from *N. gonorrhoea*. CmeR is a repressor of an RND-efflux pump, CmeABC, that is responsive to accumulation of amphipathic bile salts (286–289). Similarly, MtrR is a repressor of an RND pump, MtrCDE, the efflux of which enables gonococcal growth on mucosal surfaces that are enriched in fatty acids and bile salts. Additionally, MtrR is a repressor of a MarR family transcriptional regulator, FarR, which in turn is a repressor of the MFS efflux pump FarAB. Therefore, MtrR also functions as a positive regulator of the FarAB efflux pump which mediates resistance to antimicrobial fatty acids (233, 279, 290–292).

1.11 Rationale and hypothesis

CA-MRSA strains are particularly efficient in colonization, transmission, and causing invasive skin and soft tissue infections (6). Our laboratory strain of choice is USA300, the dominant CA-MRSA strain in the United States, accounting for 98% of MRSA infections presenting to emergency departments (293). USA300, which was isolated in September 2000, has been linked to infection outbreaks in at least 21 American states, Canada, and Europe (132). Further, this isolate is more resistant to killing by polymorphonuclear leukocytes (132). To persist on human skin, *S. aureus* must cope with uFFAs; a component of human innate defense mechanisms. Although genome sequencing and molecular typing have provided a better understanding of the pathogenic success of USA300, detailed knowledge of the specific molecular determinants, especially in regards to uFFA resistance, is still lacking (294, 295). Although *S. aureus* possess the genes coding for enzymes involved in fatty acid degradation, studies have ruled out their involvement in exogenous fatty acid utilization (214). Therefore, the only metabolic fate for exogenous uFFAs is through their incorporation into the membrane phospholipid. This incorporation step is the rate-limiting step, such that when the rate of
fatty acid import exceeds that of incorporation, excess free fatty acids accumulate in the membrane, ultimately resulting in cell death (202, 214, 216).

To better understand the factors that contribute to *S. aureus* adaptation to uFFA, our laboratory evaluated the growth of *S. aureus* USA300 in the presence of physiological concentrations of uFFA that would normally be encountered on the skin and in the anterior nares. Arsic *et al.* showed that uFFA can induce the staphylococcal proteolytic cascade (296). Additionally, Arsic *et al.* showed that unsaturated linoleic and sapienic acids are sub-inhibitory at 25 µM and bactericidal at 100 µM, whereas a 50 µM concentration caused a 10-12 hour lag phase after which bacteria resumed growth (296). It was established that the profile of secreted proteins and virulence factors is influenced by exogenous uFFAs, suggesting a possible regulatory mechanism of virulence in response to uFFAs.

To identify genes that may confer intrinsic resistance to uFFA, *S. aureus* USA300 was selected for growth at elevated concentrations of linoleic acid after which single colonies were selected for DNA isolation and genome sequencing on the Ion Torrent Platform to identify single nucleotide polymorphisms (SNP) in 7 fatty acid resistant (FAR) clones. Two of these clones contained SNP causing a His\textsubscript{121}>Tyr substitution in \textit{SAUSA300_2490} gene. Using domain enhanced lookup time accelerated BLAST, the amino acid sequence of \textit{SAUSA300_2490} had 99% homology to TFRs and structure prediction using homology modeling server, Phyre2, predicted that \textit{SAUSA300_2490} shares 99.8% amino acid sequence similarity with TFRs such as FadR, a regulator of fatty acid degradation in \textit{Thermus thermophilus}.

TFRs typically exert their effect on divergent genes. Bioinformatics analyses revealed that \textit{SAUSA300_2489}, which is divergently transcribed from \textit{SAUSA300_2490}, encodes a gene product that belongs to the Resistance-Nodulation-Cell Division (RND) superfamily of proteins, which promote proton-antiport dependent efflux mechanisms. To date, there is no evidence of a specific uFFA efflux mechanism in *S. aureus*, and since \textit{SAUSA300_2490} was found through the \textit{in vitro} selection of variants with increased resistance to linoleic acid, I speculated that this gene may contribute to uFFA resistance.
I hereafter refer to SAUSA300_2490 as farR for regulator of fatty acid resistance, and to SAUSA300_2489 as farE, for an effector of fatty acid resistance. Therefore, I hypothesize that farR functions to regulate expression of a fatty acid efflux pump encoded by farE and that a SNP in farR is sufficient to confer resistance to fatty acids. The specific aims of this thesis were to first confirm that the resistance to uFFAs in S. aureus is inducible, and examine the role of farR and farE in this induction. Second, I wanted to examine the nature of regulation that FarR exerts on farE, and define the exact operator sites that support FarR binding. Third, I aimed to examine how this novel regulatory system is linked to fatty acid detoxification mechanism both in vitro and in vivo.
1.12 Literature cited


sequence that controls expression of the ica locus in *Staphylococcus aureus* and characterization of the DNA-binding properties of IcaR. Mol Microbiol 48:889–99.


Chapter 2

2 Inducible expression of a Resistance-Nodulation-Division-Type efflux pump in *Staphylococcus aureus* provides resistance to Linoleic and Arachidonic Acids

---

2.1 Introduction

*Staphylococcus aureus* has a dichotomous relation with human hosts, being able to establish an asymptomatic commensal relationship, but also historically known as a leading cause of human infectious morbidity and mortality. Significantly, death attributed to *S. aureus* in the United States is now comparable to mortality rates for AIDS, tuberculosis, and viral hepatitis (1-3). Not surprisingly, therefore, *S. aureus* has been the subject of intensive research on mechanisms of pathogenesis, acquisition and transfer of antibiotic resistance, and efforts to identify potential vaccine antigens (4-6). Until the late 1990’s, much of this was directed towards hospital-associated strains of methicillin resistant *S. aureus* (HA-MRSA), to address the anticipated emergence of superbugs that would be resistant to all clinically useful antibiotics (7, 8). However, a new threat emerged in the late 1990’s with community acquired MRSA (CA-MRSA). Although these strains evolved in the community setting, one notorious strain known as USA300 has achieved pandemic status across North America, and is now the leading cause of *S. aureus* infections, irrespective of community or hospital origins (9, 10). This has engendered greater attention towards identifying mechanisms of *S. aureus* persistence on human hosts, and host-to-host transmission.

Approximately 25% of humans are persistently colonized by *S. aureus*, where the preferred site of colonization is the anterior nares and, among colonized individuals, the bacterium is also frequently recovered from other body sites, including the axillae, perineum, hands, chest and limbs (11). Accordingly, its ability to persist on skin is an important mediator of transmission, as underscored by the recent discovery that the hyper-transmissible USA300 strain has overcome one of the innate defense barriers of the skin through horizontal gene transfer with *S. epidermidis*, to acquire resistance to toxic polyamines that restrict the growth of other *S. aureus* strains (12, 13). Other innate defence barriers of the skin include its acidic pH, and antimicrobial fatty acids, foremost of which is sapienic acid that is released from triglycerides secreted by the sebaceous glands (14, 15). Nasal secretions also contain antimicrobial fatty acids; primarily linoleic-, arachidonic- and palmitoleic acid, or their corresponding cholesterol esters (16), and infected abscess tissue also contains abundant antimicrobial fatty acids (17, 18).
Consequently, *S. aureus* is exposed to antimicrobial fatty acids, not only during colonization, but also during infection and, as such, it is reasonable to hypothesize that it has evolved mechanisms of intrinsic resistance.

Among mechanisms that have been described, cell surface teichoic acids can selectively restrict the access of palmitoleic acid to the cytoplasmic membrane (19), and a cell surface protein IsdA that is expressed in response to iron-limiting conditions also restricts the access of palmitoleic acid, or its isomer sapienic acid to the cytoplasmic membrane (20). Others have reported that tet38, encoding a Major Facilitator Superfamily (MFS) efflux pump, promotes resistance to palmitoleic acid (21). Expression of tet38 was induced by palmitoleic acid, but not by linoleic acid, which suggested that there could be distinct mechanisms for coping with different antimicrobial fatty acids. Importantly, linoleic acid is an essential fatty acid for humans, which must be obtained from dietary sources, and is an essential precursor for synthesis of arachidonic acid. These two unsaturated fatty acids comprise a major proportion of unsaturated fatty acids in membrane phospholipid (22, 23). Therefore, the ability to sense and respond to linoleic acid could represent a specific sensory mechanism to signal colonization or infection of a human host, and yet specific mechanisms for regulating gene expression and intrinsic resistance in response to linoleic acid have not been reported.

To address this, we drew from our previous observation that exposure of *S. aureus* USA300 to a sub-inhibitory (25 µM) concentration of linoleic acid, caused a robust induction of secreted protease expression, which led to proteolytic processing of a secreted glycerol ester hydrolase, Geh (24). We subsequently noted that when *S. aureus* cultures were supplemented with a trilinolein triglyceride substrate, Geh activity quickly liberated growth inhibitory concentrations of linoleic acid (25). Moreover, 50 µM free linoleic acid imposed a 10-12h growth delay in cultures of *S. aureus* USA300, which was then followed by unimpeded exponential growth, and similar results were obtained with 50 µM trilinolein in wild type geh proficient *S. aureus* USA300, whereas growth of a geh deficient mutant was unaffected by 50 µM trilinolein (24, 25). From these observations, we hypothesized that, in addition to the induction of expression of secreted proteases, there should also be an inducible mechanism for resistance of *S. aureus* to linoleic acid.
In related studies, selection of *S. aureus* strains that were able to grow at elevated concentrations of glycopeptides led to the identification of point mutations in the *vraS* sensor of antimicrobial glycopeptides (26, 27). Therefore, we adopted a similar strategy, by conducting comparative genome sequencing of USA300 clones that were selected for their ability to initiate growth without a lag phase, when inoculated into media containing 50 µM linoleic acid. We now provide the first description of a novel gene pair *farR-farE* (fatty acid resistance) constituting divergently transcribed genes that, respectively, encode a regulator and effector of *S. aureus* resistance to linoleic and arachidonic acid.

### 2.2 Materials and methods

#### 2.2.1 Bacterial strains and growth conditions

A list of bacterial strains and plasmids that were used or constructed for this study is provided in Table 2.1. *S. aureus* cultures were maintained as frozen stocks (-80°C) in 20% glycerol, and streaked on TSB agar when required. TSB was supplemented, when required, with 10 µg/mL of erythromycin or chloramphenicol for propagation of strains bearing resistance markers. *E. coli* strains were grown on LB agar, or LB broth containing 100 µg/mL ampicillin when required. Unless otherwise stated, all cultures were grown at 37°C, and liquid cultures were incubated on an orbital shaking platform at 180 rpm.

For experimental purposes, inoculum cultures of *S. aureus* were prepared by transferring cells, from a single colony, into 13 mL polypropylene tubes containing 3 mL of TSB supplemented with antibiotic, as required, followed by overnight incubation. After determination of optical density at 600 nm (OD<sub>600</sub>), aliquots of the overnight cultures were diluted into 25 mL of medium in 125 mL flasks, to achieve an initial OD<sub>600</sub> equivalent to 0.01. To supplement media with different fatty acids, a 5 mM stock concentration was initially prepared in sterile TSB containing 1% DMSO, and then diluted into sterile TSB supplemented with 0.1% DMSO, to achieve the desired concentration of fatty acids, ranging from 5 µM to 100 µM.
<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus:</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA300 LAC</td>
<td>Community associated MRSA; wild type strain cured of resistance plasmids</td>
<td>(24)</td>
</tr>
<tr>
<td>RN4220</td>
<td>$r_k^– m_k^+$; capable of accepting foreign DNA</td>
<td>(32)</td>
</tr>
<tr>
<td>NE1393</td>
<td>Transposon insertion in SAUSA300_2490; Erm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(34)</td>
</tr>
<tr>
<td>NE2336</td>
<td>Transposon insertion in SAUSA300_2489; Erm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(34)</td>
</tr>
<tr>
<td>USA300&lt;sup&gt;farR&lt;/sup&gt;::ΦNE</td>
<td>USA300 LAC recipient of transposon from NE1393</td>
<td>This study</td>
</tr>
<tr>
<td>USA300&lt;sup&gt;farR&lt;/sup&gt;::ΦNE + pLI&lt;sup&gt;farR&lt;/sup&gt;</td>
<td>farR::ΦNE complemented with native <em>farR</em>, cloned in pLI50; Erm&lt;sup&gt;r&lt;/sup&gt;, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>USA300&lt;sup&gt;farR&lt;/sup&gt;::ΦNE + pCN&lt;sup&gt;farR&lt;/sup&gt;</td>
<td>farR::ΦNE complemented with pCN&lt;sup&gt;farR&lt;/sup&gt; for cadmium inducible expression; Erm&lt;sup&gt;r&lt;/sup&gt;, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>USA300&lt;sup&gt;farE&lt;/sup&gt;::ΦNE</td>
<td>USA300 LAC recipient of transposon from NE2336</td>
<td>This study</td>
</tr>
<tr>
<td>USA300&lt;sup&gt;farE&lt;/sup&gt;::ΦNE + pLI&lt;sup&gt;farE&lt;/sup&gt;</td>
<td>farE::ΦNE complemented with native <em>farE</em>, cloned in pLI50</td>
<td>This study</td>
</tr>
<tr>
<td>USA300&lt;sup&gt;farE&lt;/sup&gt;::ΦNE + pLI50</td>
<td>USA300&lt;sup&gt;farE&lt;/sup&gt;::ΦNE with empty pLI50 vector; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>USA300&lt;sup&gt;Δtet38&lt;/sup&gt;</td>
<td>USA300 LAC with internal deletion of <em>tet38</em> (SAUSA300_0139)</td>
<td>This study</td>
</tr>
<tr>
<td>USA300&lt;sup&gt;Δtet38-farE&lt;/sup&gt;::ΦNE</td>
<td>USA300&lt;sup&gt;Δtet38&lt;/sup&gt; recipient of *farE::ΦNE transposon insertion; Erm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
</tbody>
</table>
\textit{E. coli} DH5\textalpha 
\begin{itemize}
  \item \(\lambda^+\phi80\Delta lacZ\Delta M15\Delta(lacZYA-argF)U169\)
  \item \(\text{recA1 endA1 hsdR17(rK- mK-) supE44 thi-1}\)
  \item \(\text{gyrA relA1}\)
\end{itemize}

**Plasmids:**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLI50</td>
<td>\textit{E. coli}-\textit{S. aureus} shuttle vector</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pLI\textit{farR}</td>
<td>pLI50 with native \textit{farR} gene</td>
<td>This study</td>
</tr>
<tr>
<td>pLI\textit{farE}</td>
<td>pLI50 with native \textit{farE} gene</td>
<td>This study</td>
</tr>
<tr>
<td>pGY\textit{lux}</td>
<td>\textit{E. coli}-\textit{S. aureus} shuttle vector harboring promoterless \textit{luxABCDE} operon</td>
<td>(37)</td>
</tr>
<tr>
<td>pCN51</td>
<td>\textit{E. coli}-\textit{S. aureus} shuttle vector with \text{P}cad promoter for cadmium inducible gene expression</td>
<td>(36)</td>
</tr>
<tr>
<td>pCN51\textit{c}</td>
<td>pCN51, with \textit{ermC} cassette replaced by \textit{cat194} cassette from pRN7146</td>
<td>This study</td>
</tr>
<tr>
<td>pCN51\textit{farR}</td>
<td>pCN51\textit{c} with promoterless \textit{farR}, for cadmium inducible expression of \textit{farR}</td>
<td>This study</td>
</tr>
<tr>
<td>pKOR-1</td>
<td>\textit{E. coli}-\textit{S. aureus} shuttle vector; contains \text{P}xy\text{t} tetO; antisense \textit{secY} RNA expression</td>
<td>(38)</td>
</tr>
<tr>
<td>pKOR\textit{tet38}</td>
<td>pKOR-1 containing upstream and downstream flanking sequences for deletion of \textit{tet38}</td>
<td>This study</td>
</tr>
</tbody>
</table>
2.2.2 Selection and comparative genome sequencing of fatty acid resistant (FAR) clones

As reported previously, when an overnight culture of *S. aureus* USA300 was inoculated into fresh TSB containing 50 µM linoleic acid, there was a 10-12h lag phase, followed by unimpeded exponential growth (24). Therefore, to promote the selection of fatty acid resistant clones, seven separate flasks of *S. aureus* USA300 were subjected to two consecutive cycles of growth to stationary phase in TSB + 50 µM linoleic acid, after which, samples of each culture were plated for isolation of single colonies. Colonies from each plate were screened to identify fatty acid resistant (FAR) clones that could initiate growth without a lag phase, when inoculated into TSB + 50 µM linoleic acid. A single FAR clone was then selected from each of the seven separate biologic replicates, for comparative genome sequencing. For controls, two single colonies of USA300 were selected after two consecutive cycles of growth in TSB alone.

For comparative genome sequencing, genomic DNA was extracted from *S. aureus* using previously described protocols (28, 29). All samples for comparative genome sequencing were processed at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada; http://www.lrgc.ca) using the Ion Torrent Personal Genome Machine (PGM) (Life Technologies, Carlsbad, CA) and 316 chips. Briefly, genomic DNA was quantified using the Qubit and the Qubit dsDNA HS Assay (Life Technologies, Carlsbad, CA). Samples then underwent fragmentation, adapter and barcode ligation as per the Ion Xpress Fragment Library Kit (4469142 Rev. B), and size selection using the Pippin Prep (Sage Science, Beverly, MA). The size of the final libraries was verified using the Agilent 2100 Bioanalyzer and the High Sensitivity DNA kit (Agilent Technologies Inc., Palo Alto, CA). Barcoded libraries were pooled at equimolar concentrations, based on Qubit values, and the Template Dilution Factor (TDF) for the final pooled library was calculated using molarity determined via qPCR, with the Ion Library Quantification kit (4468802). Diluted libraries were processed as per the Ion OneTouch Template Kit (4468007, Rev. B) for automated clonal amplification, and sequenced using the Ion Express Template 200 kit (4474280), Enrichment Station and the Ion Sequencing 200 Kit (4471999, Rev. B). Sequence reads were mapped to the
genome of *S. aureus* USA300 FPR3757 (30) using CLC Genomics Workbench 7.0 (Boston, MA), and automated detection of single nucleotide polymorphisms (SNPs) was conducted using the Neighborhood Quality Standard algorithm (31).

### 2.2.3 Strain and plasmid construction

Techniques for genetic manipulation of *S. aureus* were conducted according to established guidelines (32), and as described in our previous work (24, 25, 33). The University of Nebraska transposon mutant library (34) was used as a source of transposon insertions that inactivated SAUSA300_2490 (NE1393) and SAUS300_2489 (NE2336). These were transferred into plasmid cured USA300 strain LAC, creating USA300*farR::ΦNE* and USA300*farE::ΦNE* respectively (Table 2.1). All recombinant plasmids were first constructed as shuttle vectors in *E. coli* DH5α. The integrity of plasmids isolated from *E. coli* were confirmed by restriction enzyme digestion, and nucleotide sequencing of the cloned DNA fragments prior to electroporation into *S. aureus* RN4220 as an intermediate host. From *S. aureus* RN4220, the individual plasmids were then introduced, via electroporation into *S. aureus* USA300 or isogenic derivatives as required. Primers used for PCR amplification of gene segments that were required for plasmid construction are listed in Table 2.2.

Plasmid pLI50 (35) was used to complement mutations in SAUSA300_2490 (*farR*), and SAUSA300_2489 (*farE*). To complement *farE*, a 2.8-kb fragment was amplified by PCR of genomic DNA from *S. aureus* USA300 with forward and reverse primers *farE_F1* and *farE_R1*. Similarly, a 1.2-kb product containing the native *farR* gene was amplified with primers *farR_F1* and *farR-R1*. The PCR products were digested with *KpnI* and *Sacl*, and ligated into pLI50, which had been digested with the same enzymes. To construct pCN51*farR* in which expression of *farR* is dependent on the cadmium inducible *P_{cad}* promoter, we first excised the *ermC* cassette from pCN51 by digestion with *AvrII* and *XhoI*, and replaced it with a 1.0 kb *AvrII-XhoI* fragment containing the *cat194* cassette from pRN7146 (36). The resulting pCN51c plasmid was then digested with *BamHI* and *Ascl*, and ligated to a 605 nt *BamHI-Ascl* fragment containing the promoterless *farR* gene, which was generated by PCR with primers CN*farR_F* and CN*farR_R*. To construct pGY*farE::lux*, in which expression of the luciferase operon is driven from the *farE*
promoter, a 396 bp fragment containing the intergenic segment between SAUSA300_2490 and SAUSA300_2489 (farE) was amplified with primers GY\textit{farE}\_F and GY\textit{farE}\_R, and cloned into the \textit{Bam}HI and \textit{Sal}I sites of pGY\textit{lux} (37).

A markerless in-frame deletion of \textit{tet38} (SAUSA300_0139), encoding a major facilitator efflux pump, was constructed using pKOR-1, following established protocols (25, 38). Briefly, sequences flanking the \textit{tet38} locus were amplified by PCR using primers \textit{tet38} 5'F and \textit{tet38} 5'R to generate the upstream arm, and primers \textit{tet38} 3’F and \textit{tet38} 3’R to generate the downstream arm. The upstream and downstream flanking arms were digested with \textit{Sac}II, ligated to one another, and then recombined into the temperature-sensitive pKOR-1 vector, using \textit{attB1} and \textit{attB2} sites incorporated into the flanking sequences by the respective \textit{tet38} 5'F and \textit{tet38} 3’R primers. The resulting pKOR-1\Delta\textit{tet38} vector was first passaged through \textit{S. aureus} RN4220 before being introduced into \textit{USA300} by electroporation. The correct deletion of codons 42 through 439 of the \textit{tet38} gene was confirmed by PCR and DNA sequence analysis. The resulting \textit{USA300}\Delta\textit{tet38} strain was then used as a recipient for phage transduction, using \textit{USA300}\textit{farE::\Phi}\text{NE} as a donor (Table 2.1), to create \textit{USA300}\Delta\textit{tet38-}\textit{farE::\Phi}\text{NE}. 


### Table 2.2 Primers used for construction of plasmids in Chapter 2.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>farE</strong> _F1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>cccggtaceCAGTGCAAAAGACCTCC</td>
</tr>
<tr>
<td><strong>farE</strong> _R1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>cccgagctcTGTACGGTGATCGAGTGCTTG</td>
</tr>
<tr>
<td><strong>farR</strong> _F1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>cccggtaceTGAGCAATCATACACATCCATGC</td>
</tr>
<tr>
<td><strong>farR</strong> _R1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>cccgagctcACGGACGCTAAAACAGGGATGCC</td>
</tr>
<tr>
<td>CNfarR<strong>_F&lt;sup&gt;d&lt;/sup&gt;</strong></td>
<td>cccggtccgtaactatttaACTACACAAAGGAGAAATGTGATG</td>
</tr>
<tr>
<td>CNfarR<strong>_R&lt;sup&gt;e&lt;/sup&gt;</strong></td>
<td>cccggtcgcTGAATGTGGGTAACGCTCATGAG</td>
</tr>
<tr>
<td>GYfarE<strong>_F&lt;sup&gt;f&lt;/sup&gt;</strong></td>
<td>cccggtccTGTACGGGTACGAGTGAGTCG</td>
</tr>
<tr>
<td>GYfarE<strong>_R&lt;sup&gt;g&lt;/sup&gt;</strong></td>
<td>cccggtcgcTGACATTTGTAGCAAGTGTG</td>
</tr>
<tr>
<td>tet38 5′&lt;sup&gt;h&lt;/sup&gt;F&lt;sup&gt;i&lt;/sup&gt;</td>
<td>attB1-GAAAACGGTGTCTATTCGAC</td>
</tr>
<tr>
<td>tet38 5′&lt;sup&gt;h&lt;/sup&gt;R&lt;sup&gt;j&lt;/sup&gt;</td>
<td>ggacctccggtTGAAGTCATGCAATGGCTACAG</td>
</tr>
<tr>
<td>tet38 3′&lt;sup&gt;f&lt;/sup&gt;F&lt;sup&gt;i&lt;/sup&gt;</td>
<td>ggacctccggtGTCAGCTTAATCGTTGACAC</td>
</tr>
<tr>
<td>tet38 3′&lt;sup&gt;j&lt;/sup&gt;R&lt;sup&gt;i&lt;/sup&gt;</td>
<td>attB2-CGCCACCTGTAGCTTACTTACTCAC</td>
</tr>
</tbody>
</table>

<sup>a</sup>Lower case nucleotides indicate 5′-additions, to incorporate restriction endonuclease sites for **<sup>b</sup>KpnI, <sup>c</sup>SacI, <sup>d</sup>BamHI** and stop codons (bold) in all three reading frames, **<sup>e</sup>Asci, **<sup>f</sup>BamHI, <sup>g</sup>SalI, <sup>h</sup>attB1 site GGGGACAAAGTTTGTACAAAAAAAGCGAGGCT for cloning in pKOR-1; **<sup>l</sup>SacII site; **<sup>j</sup>attB2 site GGGGACCACCTTTGTACAAAGAAGCTGGGT for cloning in pKOR-1.
2.2.4 Assay of growth and bactericidal activity

For growth and bactericidal assays, inoculum cultures were supplemented with antibiotic where required, and these cultures were then inoculated into media that lacked antibiotics to assess growth or bactericidal activity, in the presence of antimicrobial fatty acids. For growth assays, flasks containing a 1:5 ratio of flask size to medium volume, and supplemented with the indicated concentrations of fatty acid, were inoculated to an initial OD$_{600}$ of 0.01, and samples were withdrawn at hourly intervals for determination of OD$_{600}$. All cultures were grown in triplicate, or quadruplicate, as specified in individual Figure Legends. For bactericidal assays, the overnight inoculum cultures were first sub-cultured into 25 mL of fresh TSB alone, to prepare non-induced cells, or TSB containing 20 µM sub-inhibitory fatty acid, to allow for induction of intrinsic resistance mechanisms. After growth to mid-exponential phase (OD$_{600}$ = 0.5), these inoculum cultures were then inoculated into triplicate or quadruplicate flasks of fresh TSB (OD$_{600}$ = 0.01; approximately 2x10$^6$ cfu/mL) containing a 100 µM bactericidal concentration of fatty acid. The cultures were then incubated with shaking at 37°C, and aliquots were withdrawn at hourly intervals for preparation of serial dilutions in sterile TSB. Subsequently, 10 µl aliquots from each dilution were spotted in quadruplicate, on to TSB agar plates, and colonies were counted after 24h of incubation. The mean of each quadruplicate technical replicate was entered as a single data point for each flask, from which the mean and standard deviation of the biologic replicate flasks was determined.

2.2.5 Assay for uptake of $^{14}$C-linoleic acid

Assays for growth and uptake of $^{14}$C-linoleic acid were conducted according to established protocol (39), with modifications, to evaluate the influence of farE on accumulation of $^{14}$C-linoleic acid in S. aureus cells. Briefly, quadruplicate cultures of S. aureus USA300 or isogenic USA300farE::ΦNE complemented with empty pLI50 vector, or pLI/farE, were grown in TSB + 20 µM linoleic acid to an OD$_{600}$ of approximately 0.3, to allow for induction of farE. The cultures were then supplemented with an additional 50 µM dose of linoleic acid and returned to the shaker. After 30 minutes of exposure to 50 µM linoleic acid, aliquots were withdrawn, and supplemented with 0.2 µCi/mL of $^{14}$C-linoleic acid. Aliquots of 200 µL were then removed at intervals of 1, 2, 5, and 10
minutes, and samples from each replicate were simultaneously filtered onto 0.45 μM membrane filter discs using a vacuum manifold. The filters were then washed twice with 4 mL of 0.1 M phosphate buffer, pH 7.0 containing 1% Triton X-100, and after drying, were placed in scintillation vials containing 4 mL of Cytoscint scintillation cocktail (Fisher Scientific). Accumulated $^{14}$C-linoleic acid was then quantified using a Beckman LS 6500 scintillation system. Data are expressed as pMol of $^{14}$C-linoleic acid accumulated, per μg of total cell lysate protein in each sample.

2.2.6 $farE::lux$ reporter gene assays

Inoculum cultures harboring of pGY$farE::lux$ or pGY$lux$ control plasmid were subcultured into triplicate or quadruplicate flasks of TSB, or TSB supplemented with different fatty acids, to achieve an initial $OD_{600} = 0.01$. The cultures were incubated at 37°C with orbital shaking, and samples were withdrawn at hourly intervals for $OD_{600}$ determinations. For quantification of luminescence, 4 x 200-µL aliquots of each sample were added to 96 well white opaque flat bottom plates (Greiner bio-one). After supplementing each well with 20 µL of 0.1% vol/vol decanal in 40% ethanol, luminescence measurements were immediately taken on a BioTek Synergy H4 Hybrid Reader (BioTek; Winooski, VT) with 1 second of integration and a gain of 200. Data values were recorded as relative light units (RLU), corrected for background by subtraction of values recorded from cultures harbouring the empty pGY$lux$ vector. The data points were standardized for differences in growth by dividing RLU values with the recorded $OD_{600}$ of the cultures, when samples were withdrawn.

2.2.7 Data analyses

Data points for growth, viability, and luciferase reporter gene assays were plotted and analyzed using Graph Pad Prism v6.0f. Significant differences at specific time points were determined by unpaired one-tailed Student’s t-tests.
2.3 Results

2.3.1 Identification of single nucleotide polymorphism in linoleic acid resistant variants of S. aureus

The preferred site of S. aureus colonization of humans is the anterior nares, where concentrations of linoleic acid in nasal secretions can reach 40- to 50 µM (16). These values correlate with our previous work, where 50 µM linoleic acid caused a 10-12h lag phase in growth of USA300, followed by unimpeded exponential growth (24). Following up on this, we observed that when stationary phase cells from a primary culture grown in TSB + 50 µM linoleic acid were re-inoculated into the same medium, growth resumed without a lag phase (Appendix 2). To determine if this was due to the selection of genetic variants with increased resistance to linoleic acid, stationary phase cells from this second culture were plated on TSB agar for selection of single colonies. From these, we identified several that could initiate growth without a lag phase, when inoculated into TSB + 50 µM linoleic acid. Seven such fatty acid resistant (FAR) clones were subjected to comparative genome sequencing, and two of these, designated FAR6 and FAR7, had an identical single nucleotide polymorphism (SNP); a C>T transition that alters the H121 codon (CAT) to Y (TAT) in a putative transcriptional regulator encoded by SAUSA300_2490 (30). FAR6 had a second SNP in a pyruvate oxidase encoded by cidC. Therefore, we focused on FAR7, which had just one SNP in SAUSA300_2490, and re-sequencing of this gene in USA300 and FAR7 confirmed the unique SNP in FAR7.

2.3.2 Description of the farE-farR locus

We hypothesize that SAUSA300_2490, and a divergently transcribed gene SAUSA300_2489, respectively comprise a regulator and effector gene pair that we have designated as farR and farE, to denote predicted functions as a regulator and effector of fatty acid resistance. These assignments are supported by bioinformatics analyses. farR encodes a 182 amino acid protein, with an N-terminal TetR family DNA binding domain (2.33e^-04), and overall similarity to the AcrR cluster of orthologous groups of proteins (6.52e^-09). In Gram-negative bacteria, AcrR regulators control expression of efflux pumps belonging to the AcrB family, which are often encoded by divergently transcribed genes, as with acrR-acrABC in E. coli (40) and orthologous mtrR-mtrCDE arrangement in N.
gonorrhoeae (41). Similarly, farE is divergently transcribed from farR and encodes an 822 amino acid protein that is annotated as a drug exporter of the resistance-nodulation-division (RND) superfamily (30), to which AcrB and orthologous efflux pumps are also assigned (42). Genome annotation also assigns FarE to the MMPL family of proteins, on the basis of homology to large membrane proteins of Mycobacterium tuberculosis, that transport mycolic acids to the cell surface (43). Using protein structural modeling programs HHpred and Phyre2 (44, 45), FarR was predicted with greater than 99% confidence, to resemble known AcrR family regulators, including PfmR and FadR of Thermus thermophilus, which control expression of genes involved in fatty acid synthesis and metabolism (46, 47), and MtrR; an efflux pump regulator of Neisseria gonorrhoeae (41, 48). Likewise, 80% of the FarE amino acid sequence was modeled with 100% confidence, on the structure of AcrB from E. coli (49).

2.3.3 farR is required for inducible resistance to linoleic acid

We hypothesized that farR should regulate expression of farE in response to antimicrobial fatty acids, which was addressed by constructing a farE::lux reporter, where expression of the lux operon is under transcriptional control of the farE promoter. When USA300-pGYfarE::lux was cultured in TSB, there was a modest peak of luciferase activity in early exponential growth, which quickly dissipated (Fig. 2.1). However, in TSB + 20 µM linoleic acid, luciferase activity was strongly induced in early exponential phase cells, and again dissipated as the cells progressed towards stationary phase. Importantly, no induction was observed in USA300farR::ΦNE cells. Although USA300farR appeared to exhibit superior growth to wild type USA300 in TSB + 20 µM linoleic acid (Fig. 2.1), our further analysis on this phenomenon uncovered that it reflects a growth penalty that is imposed on USA300 by forced expression of the luxABCDE genes. This was evident from a growth comparison of USA300 harboring either pGYfarE::lux or empty pGYlux, in TSB + 20 µM linoleic acid, where cells carrying pGYfarE::lux exhibited significantly slower growth compared to USA300 with empty vector (Appendix 3).

These assays suggested that USA300 should exhibit inducible resistance to the antimicrobial activity of linoleic acid. It was previously reported that exponential phase
cells of *S. aureus* were significantly more sensitive to the bactericidal activity of antimicrobial fatty acids, relative to stationary phase cells (17), which we confirmed in a preliminary experiment (Appendix 4). Therefore, to assess inducible resistance, USA300 and USA300*farR::ΦNE* were grown to mid-exponential phase in TSB (non-induced), or TSB + 20 μM linoleic acid (induced), and then diluted to 10^6 cfu/mL in fresh TSB containing 100 μM linoleic acid. Non-induced USA300 suffered a > 3-log loss of viability after 1h of exposure to 100 μM linoleic acid (Fig. 2.2A), while the induced cells retained significantly greater viability at all time points, such that there was only an approximate 40-fold loss of viability after 5h. Furthermore, the induced USA300*farR::ΦNE* cells exhibited a significantly greater loss of viability compared to induced USA300, after 2h and onwards. Although the induced USA300*farR::ΦNE* cells initially retained significantly greater viability compared to non-induced USA300, they exhibited a progressive loss of viability, such that after 4h of exposure, the remaining viable cells did not significantly differ from non-induced USA300 cells.
Figure 2.1 Linoleic acid induces expression of \textit{farE}. Growth (OD$_{600}$; open symbols) and relative luminescence units (RLU/OD; closed symbols) of USA300 and USA300\textit{farR::ΦNE}, harboring the pGY\textit{farE::lux} reporter vector, are charted. USA300 was grown in TSB or TSB + 20 µM linoleic acid (LA); USA300\textit{farR::ΦNE} was grown in TSB or TSB + 20 µM linoleic acid. Each value represents the mean and standard deviation of three separate cultures, and each culture was subjected to quadruplicate luminescence readings at each time point.
Viability in 100 μM linoleic acid

Figure 2.2 Sensitivity of USA300 and USA300\textit{farR::\Phi NE} to the bactericidal activity of 100 μM linoleic acid. A. USA300 or USA300\textit{farR::\Phi NE} challenge cells were grown to mid-exponential phase in TSB or TSB + 20 μM linoleic acid and then diluted to 10⁶ cfu/mL in TSB containing 100 μM linoleic acid, followed by monitoring of viability at hourly intervals. B. USA300\textit{farR::\Phi NE} was complemented with empty pLI50 vector or pLI\textit{farR} and assayed for viability in 100 μM linoleic acid, after initial growth in TSB + 20 μM linoleic acid. All data points represent the mean ± standard deviation, of viability determinations from quadruplicate cultures. Significant differences in viability at each time point were determined by unpaired one-tailed Student’s t-test; ***; P < 0.001; **; P < 0.01; *; P < 0.05; ns, nonsignificant.
To validate a role for farR, USA300farR::ΦNE was complemented with empty pLI50, or pLIfarR harboring farR and its native promoter, to determine whether pLIfarR could restore inducible resistance. Accordingly, when pre-induced by growth in 20 µM linoleic acid, USA300farR::ΦNE + pLIfarR retained significantly greater viability after 2h of exposure to 100 µM linoleic acid, compared to USA300farR::ΦNE + pLI50 (Fig. 2.2B). Nevertheless, pLIfarR did not appear to restore the level of inducible resistance to that of wild type USA300, which retained approximately 10^5 cfu/mL viable cells after 5h of exposure (Fig. 2.2A). We reasoned that this could be due to two variables; first, that farR might be expressed at a high level from its native promoter on a multi-copy plasmid, and second, that the FarR protein could engage nucleotide sequences on pLIfarR, which contained the entire farE-farR intergenic segment, and these in trans interactions could limit the ability of FarR to regulate farE on the chromosome. To overcome these limitations, we expressed farR using the cadmium inducible P_cad promoter, and observed an approximate 100-fold difference in viability when USA300farR::ΦNE + pCNfarR cells were exposed to 100 µM linoleic acid, in the presence or absence of 10 µM cadmium (Appendix 5). Cumulatively, these data support the contention that farR is required to manifest an inducible resistance phenotype in S. aureus USA300.

2.3.4 farE contributes to persistence and growth of S. aureus in the presence of linoleic acid

We previously established that USA300 could grow in TSB containing 25 µM linoleic acid, whereas 50 µM linoleic acid imposed a 10-12h lag phase. Our current reporter gene assays also established that farE was induced by growth in TSB containing 20 µM linoleic acid. Therefore, we expected that farE would be required to support growth of S. aureus USA300 at a 25 µM upper threshold of linoleic acid, and that induction of farE would confer protection against challenge of S. aureus with a 100 µM bactericidal concentration. To address the growth requirement, USA300 or USA300farE::ΦNE were cultured in TSB containing 5, 10, 20, or 25 µM linoleic acid. USA300 was not adversely affected by 5- or 10 µM linoleic acid, but exhibited slower growth in 20- or 25 µM linoleic acid (Fig. 2.3A). Comparatively, USA300farE::ΦNE exhibited similar behavior at 5, 10 and 20 µM linoleic acid, but was unable to initiate growth over an 8h incubation...
in 25 µM linoleic acid (Fig. 2.3B). Furthermore, when USA300farE::ΦNE was complemented with pLIfarE, we observed growth restoration not only in 25 µM linoleic acid (Fig. 2.3B), but also in up to 100 µM linoleic acid (Fig. 2.3C); compare this to wild type USA300, which was unable to grow in 50 µM linoleic acid (Fig. 2.3C).

To ensure that the role of farE was not dependent on factors that are uniquely associated with the CA-MRSA strain USA300 genetic background, we transduced farE::ΦNE into S. aureus SH1000, which is a methicillin susceptible laboratory strain that has the same multi-locus sequence type (MLST) as USA300 (50). Although SH1000 exhibited somewhat greater intrinsic resistance to linoleic acid, as evident from its ability to grow in TSB + 50 µM linoleic acid, SH1000farE::ΦNE exhibited an extended lag phase, with no obvious growth over 6h (Fig. 2.3D). Therefore, farE promotes growth of both MRSA and MSSA strains at elevated concentrations of linoleic acid.

To evaluate the role of farE in promoting inducible resistance, USA300 and USA300farE::ΦNE were grown in TSB alone, or TSB containing 20 µM linoleic acid, prior to subculture into 100 µM linoleic acid (Fig. 2.4). Consistent with farE not being appreciably expressed in non-induced cells, the non-induced USA300 and USA300farE::ΦNE cultures both suffered a rapid loss of viability on exposure to 100 µM linoleic acid. However, when the cells were grown under inducing conditions prior to challenge with 100 µM linoleic acid, USA300 exhibited only a 10- to 40-fold loss of viability over 5h, and retained significantly greater viability at all time points, compared to USA300farE::ΦNE. Interestingly, the induced USA300farE::ΦNE challenge cells still retained significantly greater viability compared to non-induced USA300, which suggests that factors in addition to farE may also promote inducible resistance. Cumulatively, these data confirm that farE contributes to the inducible resistance of S. aureus to the bactericidal activity of 100 µM linoleic acid, and is also required to support growth in as low as 25 µM linoleic acid. It further appears that resistance is proportional to farE expression, as suggested by the ability of pLIfarE to support growth of USA300farE::ΦNE at concentrations of linoleic acid that could not be tolerated by USA300 (Fig. 2.3C).
A. USA300

B. USA300\textit{farE::\Phi NE}

C. USA300 and USA300\textit{farE::\Phi NE}

D. SH1000 and SH100\textit{farE::\Phi NE}

\begin{align*}
\text{OD}_{600} & \text{vs. Time (h)} \\
& 0 \quad 2 \quad 4 \quad 6 \quad 8
\end{align*}
Figure 2.3 Mutation of \textit{farE::\Phi NE} enhances sensitivity of \textit{S. aureus} to toxicity of linoleic acid. Growth of USA300 (A) or USA300\textit{farE::\Phi NE} (B) in TSB supplemented with 5 \( \mu \text{M} \), 10 \( \mu \text{M} \), 20 \( \mu \text{M} \) or 25 \( \mu \text{M} \) linoleic acid, and that of USA300\textit{farE::\Phi NE + pLIfarE} in TSB + 25 \( \mu \text{M} \) linoleic acid. C. Growth of USA300 or USA300\textit{farE::\Phi NE} in TSB + 50 \( \mu \text{M} \) linoleic acid and growth of USA300\textit{farE::\Phi NE + pLIfarE} in 50 \( \mu \text{M} \) or 100 \( \mu \text{M} \) linoleic acid. D. Growth of \textit{S. aureus} SH1000 or SH1000 \textit{farE::\Phi NE} in TSB + 50 \( \mu \text{M} \) linoleic acid. Each data point represents the mean of triplicate (A, C and D) or quadruplicate (B) cultures.
Figure 2.4 Sensitivity of USA300 and USA300\textit{farE::\PhiNE} cells to the bactericidal activity of 100 µM linoleic acid. Cells of USA300 or USA300\textit{farE::\PhiNE} were exposed to 100 µM linoleic acid after growth to mid-exponential phase in TSB or TSB + 20 µM linoleic acid. Each data point represents the mean of quadruplicate cultures. \( P \) values are indicated by asterisks where **, \( P < 0.01 \); ***, \( P < 0.001 \); ns, non-significant.
2.3.5 The FAR7 clone exhibits increased expression of farE

FAR7 is distinguished from USA300 by a SNP in farR that changes H₁₂₁ to Y in the gene product. This clone was selected for its ability to grow without a lag phase in TSB + 50 µM linoleic acid, and our data suggest that this should be due to increased expression of farE, as a consequence of the SNP in farR. This was confirmed by conducting farE::lux reporter gene assays, in both USA300 and FAR7 (Fig. 2.5). When grown in TSB, FAR7 exhibited significantly greater luciferase activity compared to USA300, and during growth in TSB + 20 µM linoleic acid, the luciferase activity in FAR7 significantly exceeded that of USA300. Therefore, the SNP that causes a H₁₂₁Y substitution in FarR results in a constitutive level of farE expression during growth in TSB, and permits a significantly greater induced level of expression, than could otherwise be achieved in USA300.

2.3.6 An H₁₂₁Y substitution in FarR is sufficient for increased resistance to linoleic acid

Since USA300 and FAR7 are differentiated on the basis of a SNP that causes a H₁₂₁Y substitution in FarR, we expected that this alone would be sufficient to promote increased resistance to linoleic acid. Accordingly, although FAR7 and USA300 exhibited no difference in growth when cultured in TSB, FAR7 was uniquely able to grow in TSB + 100 µM linoleic acid (Fig. 2.6A). In bactericidal assays, non-induced USA300 and FAR7 both suffered a similar rapid loss of viability when exposed to 100 µM linoleic acid (Fig. 2.6B). Therefore, although there is some constitutive expression of farE during growth of FAR7 in TSB, this is not sufficient to promote resistance to 100 µM linoleic acid. However, when the assay was conducted with cells grown under inducing conditions, FAR7 did not exhibit any significant loss of viability over 5h of exposure to 100 µM linoleic acid, and exhibited significantly greater retention of viability from 3- to 5h, compared to USA300 (Fig. 2.6B). These observations are consistent with our farE::lux assays, where FAR7 exhibited a significantly higher induced level of farE expression compared to USA300, and support the contention that increased expression of farE correlates with increased resistance.
Figure 2.5 The FAR7 SNP causes enhanced induction of farE expression. The cultures were grown in TSB or TSB + 20 µM linoleic acid as indicated. Data are expressed as relative luminosity units (RLU), standardized to one OD$_{600}$ unit. Values represent the mean of four replicates from each of four independent cultures. Measurements were taken from triplicate cultures when OD$_{600}$ values reached approximately 0.5, and $P$ values are indicated by asterisks where *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. 
Figure 2.6 FAR7 is more resistant than USA300 to linoleic acid. 

A. Growth analysis of USA300 and FAR7 cultured in TSB or TSB + 100 µM linoleic acid. 

B. Bactericidal activity of 100 µM linoleic acid measured with USA300 or FAR7 challenge cells, prepared by growth to mid-exponential phase in TSB or TSB + 100 µM linoleic acid. Each data point represents the mean of triplicate cultures. 

$P$ values for comparison of induced USA300 and induced FAR7 cells are indicated by asterisks where ***, $P < 0.001$. 

---

**Legend:**
- Open circle: USA300; TSB
- Filled circle: USA300; TSB + 100 µM LA
- Open square: FAR7; TSB
- Filled square: FAR7; TSB + 100 µM LA
To further define the impact of the H121Y substitution, USA300\textit{farR}:\PhiNE was transformed with pLI50 harboring wild type \textit{farR}, or the variant \textit{farR7} allele derived from FAR7. With no complementation, USA300\textit{farR}:\PhiNE exhibited no growth over an 8h incubation in TSB + 50 \textmu M linoleic acid, and cells complemented with wild type pLI\textit{farR} were also unable to grow (Fig. 2.7). However, cells complemented with the variant \textit{farR7} allele acquired the ability to grow in 50 \textmu M linoleic acid, and also at a reduced rate in 100 \textmu M linoleic acid. Therefore, a SNP that introduces a H121Y substitution in FarR is alone sufficient to confer increased resistance of \textit{S. aureus} towards linoleic acid, presumably due to increased expression of \textit{farE}.

### 2.3.7 Role of \textit{farE} in resistance to other uFFA

Although \textit{farE} is induced by and promotes resistance to linoleic acid, \textit{S. aureus} would be exposed to a varying diversity and abundance of free fatty acids, dependent on the context within the human body. In a tissue abscess, pus contains high concentrations of unsaturated free fatty acids, which could be derived from triglyceride (18, 51), or human cell membrane phospholipid, where the major unsaturated fatty acids, oleic (C18:1), linoleic (C18:2), and arachidonic acid (C20:4), each comprise approximately 13- to 15% of the total fatty acid content (22, 23). Conversely, although sapienic acid or its isomer palmitoleic acid (C16:1) do not comprise a major proportion of the fatty acid profile of phospholipid, sapienic acid is the major unsaturated fatty acid in human sebum, both as free fatty acid, and in sebum triglyceride (14, 52). Therefore, to better understand the biological role of \textit{farE}, we evaluated the specificity of \textit{farE} induction by these different fatty acids, and the extent to which \textit{farE} confers resistance to other fatty acids.

To evaluate the specificity of induction, USA300-pGY\textit{farE}:\lux was grown to an OD_{600} of ~ 0.5 in TSB, or TSB supplemented with 20 \textmu M fatty acid, followed by assay of luciferase activity (Fig. 2.8A). There were significant differences in the ability of different 18-carbon chain length fatty acids to induce \textit{farE}:\lux, such that no induction was observed with saturated stearic acid (C18:0), or oleic acid (C18:1), while linoleic acid (C18:2) was a strong inducer. Strikingly, arachidonic acid (C20:4) promoted a significantly higher level of expression compared to linoleic acid, while linolenic acid (C18:3), together with palmitoleic acid (C16:1) and its isomer sapienic acid, each
facilitated an intermediate level of expression, which was significantly greater than that of TSB alone, but significantly less than that of linoleic and arachidonic acid.

Consistent with the modest induction by 20 μM palmitoleic acid, when a bactericidal assay was conducted with USA300 and USA300\textit{farE::\Phi NE} cells that were pre-induced by growth in 20 μM palmitoleic acid, there were no significant differences in retention of viability after exposure to 100 μM palmitoleic acid (Fig. 2.8B). However, when this assay was performed with arachidonic acid, USA300 retained significantly greater viability after 2h of exposure, compared to USA300\textit{farE::\Phi NE} (Fig. 2.8B). Therefore, \textit{farE} appears to have a primary role in mediating resistance to linoleic- and arachidonic acid, which are the most effective inducers of \textit{farE} expression.

Although \textit{farE} did not promote resistance to palmitoleic acid, we nevertheless observed a significant induction of expression by 20 μM palmitoleic acid (Fig. 2.8A), in addition to which, FAR7 was able to grow in TSB containing 50 μM palmitoleic acid, whereas USA300 could not (Appendix 6). This suggested that \textit{farE} could still promote resistance to palmitoleic acid, if expressed at a sufficiently high level. Furthermore, it was recently reported that \textit{tet38}, which encodes a major facilitator superfamily efflux pump, was induced by palmitoleic acid and contributed to resistance (21). Therefore, we considered that one efflux pump might compensate for loss of another, which could obfuscate the phenotype of USA300\textit{farE::\Phi NE} when tested with palmitoleic acid. To address this, we constructed a markerless \textit{Δtet38} mutation in USA300, which was assayed for growth in TSB + 25 μM or 40 μM palmitoleic acid. The higher concentration imposed a slower growth rate, as evident from a time of 5h being required for USA300 to achieve an \textit{OD}_{600} = 0.5, compared to approximately 3h in 25 μM palmitoleic acid (Fig. 2.9). Nevertheless, there were no discernible differences in growth between USA300, and the individual USA300\textit{Δtet38} or USA300\textit{farE::\Phi NE} mutants, or combined USA300\textit{Δtet38-farE::\Phi NE} double mutant. Therefore, neither \textit{farE} nor \textit{tet38} exerted a significant impact on resistance to palmitoleic acid under the conditions that we have tested.
Figure 2.7 The variant farR7 allele, but not wild type farR, enables the ability of USA300farR::ΦNE to grow at inhibitory concentrations of linoleic acid. USA300 was grown in TSB + 25 µM linoleic acid, USA300farR::ΦNE was grown in 25 µM or 50 µM linoleic acid, USA300farR::ΦNE + pLIfarR was grown in 50 µM linoleic acid, and USA300farR::ΦNE + pLIfarR7 was grown in 50 µM or 100 µM linoleic acid. All data points represent the mean of triplicate cultures.
A. *farE::lux* Activity

- TSB
- Stearic (18:0)
- Oleic (18:1)
- Linoleic (18:2)
- Linolenic (18:3)
- Sapienic (16:1)
- Arachidonic (20:4)
- Palmitoleic (16:1)

B. Viability after 3h exposure to 100 µM fatty acid

- C18:2
- C20:4
- C16:1

Viability (cfu/mL)

- USA300
- USA300 *farE::ΦNE*
Figure 2.8 Influence of different antimicrobial fatty acids on induction of farE, or viability of *S. aureus* USA300 and USA300farE::ΦNE. A. Quantification of pGYlux::farE dependent luciferase activity in *S. aureus* USA300 grown to OD$_{600}$ = 0.5 in TSB alone, or TSB supplemented with 20 µM of fatty acid, as indicated. Each value represents the mean of quadruplicate measurements, from each of four replicate cultures. *P* values indicate significant differences compared to growth in TSB alone, or significant difference between linoleic and arachidonic acid. B. Bactericidal activity of 100 µM linoleic acid (C18:2), arachidonic acid (C20:4), or palmitoleic acid (C16:1) towards USA300 or USA300farE::ΦNE cells. The inoculum cultures were grown to OD$_{600}$ = 0.5 in TSB supplemented with 20 µM of the respective fatty acids, prior to challenge with a 100 µM bactericidal concentration. Asterisks indicate *P*-values of significant differences between USA300 and USA300farE::ΦNE. Each value represents the mean of viability determination from quadruplicate cultures. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ns, nonsignificant.
Figure 2.9 Effect of farE::ΦNE and Δtet38 mutations on growth of S. aureus in the presence of 25 µM or 40 µM palmitoleic acid (PA). USA300, USA300Δtet38, USA300Δtet38, and USA300Δtet38-farE::ΦNE were grown in TSB supplemented with 25 µM or 40 µM palmitoleic acid (PA), as indicated. The dotted line with arrow depicts the time of growth at which OD_{600} = 0.5.
2.3.8 Inactivation of \textit{farE} promotes increased uptake of $^{14}$C-linoleic acid

Although many bacteria can derive energy from exogenous fatty acids through an inducible $\beta$-oxidation pathway (53), \textit{S. aureus} lacks this ability, and its primary means of coping with exogenous fatty acids is through incorporation into phospholipid (19, 39, 54, 55). Since our data suggest that FarE promotes efflux of fatty acids, we expected that inactivation of \textit{farE} would promote increased uptake of exogenous fatty acid. Prior to quantifying uptake of $^{14}$C-linoleic acid, we first conducted a mock assay, to evaluate the ability USA300 and USA300\textit{farE::ΦNE} to recover from exposure to an abrupt increase in the concentration of linoleic acid. Cultures were grown to OD$_{600} = 0.3$ in TSB supplemented with sub-inhibitory 20 $\mu$M linoleic acid, to allow for induction of \textit{farE} in USA300, and the cells were then challenged with a 50 $\mu$M dose of linoleic acid, followed by monitoring of OD$_{600}$. After 30 minutes, each of USA300, USA300\textit{farE::ΦNE + pLI50}, and USA300\textit{farE::ΦNE+ pLIfarE} exhibited evidence of continued growth (Fig. 2.10A). However, beyond 30 minutes, growth of USA300\textit{farE::ΦNE+ pLI50} was severely impaired, whereas USA300 continued to grow, and USA300\textit{farE::ΦNE+ pLIfarE} exhibited superior recovery. These data confirm that \textit{farE} contributes to the ability of \textit{S. aureus} USA300 to recover from an abrupt increase in the concentration of exogenous linoleic acid, and that conditions of the assay were not bactericidal.

We next wished to address the question of whether FarE was responsible for actively extruding linoleic acid from the \textit{S. aureus} cell. To do this, we performed uptake assays using $^{14}$C-linoleic acid. We performed these assays on cells that were treated the same as for the growth experiments described in Figure 2.10A, and cultures were supplemented with $^{14}$C-linoleic acid, 30 minutes after challenge with 50 $\mu$M linoleic acid. Strikingly, USA300\textit{farE::ΦNE} complemented with pLI\textit{farE} exhibited the least accumulation of $^{14}$C-linoleic acid, while USA300\textit{farE::ΦNE} harboring the empty pLI50 vector exhibited the greatest accumulation, and wild type USA300 exhibited intermediate accumulation (Fig. 2.10B). Importantly, this reflected an inverse correlation between recovery of growth after exposure to 50 $\mu$M linoleic acid, and accumulation of $^{14}$C-linoleic acid. Specifically, USA300\textit{farE::ΦNE + pLIfarE} exhibited the least accumulation of $^{14}$C-linoleic acid, and
its growth was not adversely affected, whereas USA300farE::ΦNE + pL150 exhibited the greatest accumulation and its growth was severely impaired, while wild type USA300 exhibited intermediate growth and accumulation kinetics. These data support the contention that FarE mediated efflux of unsaturated free fatty acids is required to support growth of \textit{S. aureus} at elevated concentrations of antimicrobial fatty acid.
Figure 2.10 Growth (A) and uptake of $^{14}$C-linoleic acid (B) following exposure of *S. aureus* USA300 and USA300$\text{farE}::\Phi\text{NE}$ to an increase in concentration of linoleic acid. A. quadruplicate cultures of USA300, USA300$\text{farE}::\Phi\text{NE} + \text{pLI50}$, or USA300$\text{farE}::\Phi\text{NE} + \text{pLI-farE}$ were grown in TSB + 20 µM linoleic acid to an OD$_{600}$ of approximately 0.2 to 0.3. The cultures were then supplemented with an additional 50 µM dose of linoleic acid, and growth (OD$_{600}$) was measured after 30 minutes, and then at hourly intervals. When this experiment was conducted for the purpose of quantifying uptake of $^{14}$C-linoleic acid (B), the cultures were supplemented with 0.20 µCi/mL of $^{14}$C-linoleic acid at t = 30 minutes, and aliquots of culture were processed for quantification of $^{14}$C-linoleic acid uptake at intervals of 1, 2, 5, and 10 minutes. Each data point represents the mean and standard deviation of quadruplicate samples.
2.4 Discussion

Through comparative genome sequencing of *S. aureus* USA300 variants that were selected for enhanced resistance to linoleic acid, we identified a regulator of fatty acid resistance, *farR*, and an effector of fatty acid resistance *farE*, which to our knowledge, is the first description of a dedicated and inducible mechanism of *S. aureus* resistance to antimicrobial fatty acids. These genes bear a similarity to the *acrR* and *acrB* paradigm in *E. coli*, where *acrR* and *acrB* were discovered through *in vitro* selection of acriflavine-resistant mutants, which mapped to the *acr* locus (40, 56, 57). The emergence of antibiotic resistance in Gram-negative bacteria has also been attributed to the *in vivo* selection of mutations in the transcriptional repressor *acrR*, which promote increased expression of the efflux pump encoded by *acrB* (58-60). Similarly, we discovered *farR* through *in vitro* selection of USA300 variants with increased resistance to linoleic acid.

As with many proteins that possess an N-terminal TetR DNA binding domain, protein structural modeling and homology searches indicate that FarR belongs to the TetR/AcrR family of regulators, while FarE belongs to the RND-family of multidrug efflux pumps, which include AcrB.

In addition to our own work which support a role for FarE as an efflux pump, other researchers using a different approach with *S. aureus* COL, demonstrated that an amino acid substitution in FarE (SACOL2566) promotes resistance to a newly described oxadiazole family of antibiotics (61). In *E. coli*, polymorphisms that cause amino acid substitutions in AcrB can also accrue during *in vitro* selection of strains that are resistant to fluoroquinolone antibiotics (62), and in these examples, it is likely that resistance is due to amino acid substitutions that expand the substrate specificity of the efflux pump (61, 62). However, although AcrB family efflux pumps have been most extensively characterized as mediators of multiple drug resistance, we contend that the primary function of FarE is to promote efflux of antimicrobial fatty acids that would be encountered during colonization, or within a tissue abscess. This is consistent with the belief that members of the AcrB family, which are encoded by the core genome, evolved to promote efflux of host-derived toxic compounds, including bile salts and fatty acids (63-68).
It is especially significant that expression of farE was most strongly induced by linoleic and arachidonic acid. Since S. aureus cannot synthesize unsaturated fatty acids (69), our data suggest that farE is induced as part of a signaling pathway that is activated by host-specific unsaturated free fatty acids. In other work, bactericidal assays conducted with human nasal secretions established that cholesterol esters of linoleic- and arachidonic acid were the principal bactericidal components towards Pseudomonas aeruginosa, which does not colonize the nose, but did not affect viability of S. aureus (16), and linoleic acid is also the principal antimicrobial fatty acid in homogenates of murine tissue abscesses (18, 51). Although arachidonic acid was not identified as a major fatty acid in abscess homogenates, it is a major unsaturated fatty acid in erythrocyte and leukocyte membrane phospholipid (23, 70), from which it is released by phospholipases at sites of infection, and rapidly converted to inflammatory mediators (71). Therefore, the induction of farE in response to linoleic- and arachidonic acid may represent an evolutionary feature that contributes to the success of S. aureus as a human pathogen.

Our observations are consistent with a requirement for FarE in maintaining membrane homeostasis when S. aureus is exposed to host-derived antimicrobial unsaturated free fatty acids. Since S. aureus cannot degrade exogenous fatty acids through β-oxidation, its primary means of coping with exogenous fatty acids is through incorporation into membrane phospholipid, which involves a novel fatty acid kinase pathway, whereby phosphorylated fatty acid is directly incorporated into glycerol-3-phosphate (54, 55). This in itself may represent a primary means of detoxifying long chain unsaturated free fatty acids, which promote loss of membrane integrity and cell death if allowed to accumulate in the cytoplasmic membrane (19). Importantly, S. aureus cannot synthesize unsaturated fatty acids, and maintains membrane fluidity through synthesis of branched chain fatty acids; primarily anteiso-C15 (69). From these considerations, we can envision two scenarios whereby FarE would be required under such conditions.

First, although some bacteria cease the de novo synthesis of fatty acids when provided with an exogenous supply of unsaturated fatty acids, this does not occur in S. aureus, which continues to synthesize fatty acids (72). However, under such conditions, there is reduced incorporation of endogenously synthesized anteiso-C15 into phospholipid, likely
due to displacement or competition from the exogenous unsaturated fatty acid (72, 73). Consequently, it is likely that unutilized metabolites will accumulate, which could be dealt with through an efflux mechanism, and at least one study has proposed that the primary function of an RND family efflux pump is to promote efflux of fatty acids that are replaced as a result of membrane damage or phospholipid turnover (74). Second, although incorporation of unsaturated fatty acids into phospholipid may comprise an effective means of detoxification, it would also promote an increase in membrane fluidity which if too severe, would compromise membrane function. In this context, we note from our analysis of uptake of $^{14}$C-linoleic acid, that USA300$\text{farE}::\Phi\text{NE}$ cells exhibited significantly greater uptake of $^{14}$C-linoleic acid compared to wild type USA300 (Fig. 2.10B). Therefore, although growth of USA300$\text{farE}::\Phi\text{NE}$ cells was impaired under these conditions (Fig. 2.10A), it continued to accumulate $^{14}$C-linoleic acid, which suggests that there is sufficient metabolic capacity to incorporate unsaturated fatty acid into phospholipid, at a level that is beyond the tolerance for proper membrane function. Consequently, FarE function could also be required under such conditions, to ensure that incorporation of unsaturated fatty acid into phospholipid does not exceed a level of tolerance for membrane fluidity.

Although our data supported a role for $\text{farE}$ in mediating resistance to linoleic and arachidonic acid, it did not confer resistance to palmitoleic acid, which is consistent with their being distinct mechanisms for resistance to unsaturated fatty acids of 16- and 18-carbon chain length. First, $\text{S. aureus}$ exhibits a differential capacity to incorporate exogenous unsaturated 16- or 18-carbon fatty acids into membrane phospholipid. Oleic acid (C18:1) is directly incorporated into phospholipid (72), but palmitoleic acid must first be extended by the $\text{S. aureus}$ fatty acid biosynthesis machinery, in a rate limiting step, to produce C18:1, which is then incorporated into phospholipid (19). Perhaps due to the less efficient incorporation of C16:1 fatty acids into phospholipid, $\text{S. aureus}$ has evolved some capacity to exclude entry of palmitoleic and sapienic acid into the cytoplasm, due to cell surface teichoic acids and the low iron-induced cell surface protein IsdA, which function as a filtering mechanism to restrict penetration through the cell wall (19, 20). Others also reported that a major facilitator superfamily efflux pump encoded by $\text{tet38}$ promoted resistance to palmitoleic acid (21), and although we were not able to
confirm this through construction of a USA300Δtet38 deletion mutant, it may be that tet38 functions in a strain specific context.

Further relevant to these considerations, expression of tet38 was induced primarily by palmitoleic acid, and much less effectively by linoleic acid, whereas we observed the opposite response for induction of farE. Importantly, with our identification of a SNP in farR that promotes increased expression of farE, we have provided the first mechanistic description of an efflux pump that is specifically induced in response to antimicrobial fatty acids in S. aureus, and at a broader level, in Gram-positive bacteria. FarR belongs to the TetR/AcrR family of transcriptional regulators, which usually repress transcription of divergent genes, by virtue of an N-terminal DNA binding domain that recognizes a specific operator site in the promoter segment of a target gene, and the affinity of this interaction is modulated by a C-terminal domain that binds a small inducing ligand (75, 76). In a relevant example, FadR of Thermus thermophilus represses expression of genes required to degrade fatty acids, which are de-repressed upon binding of an acyl-CoA ligand to FadR (46). However, although farE is induced by antimicrobial fatty acids, we cannot yet conclude that farR is alone sufficient to regulate farE. If FarR functioned strictly as a repressor, then inactivation of farR should have caused de-repression of farE. However, this was not observed, and farR was in fact needed for induction of farE (Fig. 2.1). Conversely, FAR7 exhibited a constitutive measure of farE expression, attributed to the H121Y substitution in FarR, which also conferred a significantly higher induced level of farE expression than could be achieved in wild type USA300 (Fig. 2.8B).

As this substitution is not within the N-terminal DNA binding domain, which spans amino acids 28-61 of FarR, it should not directly affect the DNA binding function. However, in a potentially related example, FadR represses expression of genes required for β-oxidation of fatty acids, and the conformation of amino acids 106-119 in the C-terminal domain underwent a significant shift on binding of fatty acid, including R109, which had an important role in maintaining the DNA-binding affinity, even though it is not within the N-terminal DNA binding domain (77). Therefore, the H121Y substitution in FarR could still affect the function of the N-terminal DNA binding domain, or alternately...
it may affect the ability of FarR to form functional oligomers; typically dimers or tetramers, which is another characteristic trait of the TetR family of regulators (75, 78).

Although most TetR regulators repress expression of divergently transcribed genes (75, 78), our observation that FarR is required for induction of farE is not unprecedented, and FarR may resemble a limited number of TetR regulators that trigger a broader cellular response to environmental insults (78-83). In one such example, the SczA metal ion-dependent transcriptional regulator of Streptococcus pneumoniae (82) binds to a specific operator site to repress transcription of a target gene in the absence of zinc, but when zinc is present, it binds to a different DNA segment upstream of the regulated gene to activate transcription. Alternatively, FarR may still function as repressor of farE in the absence of inducer, and then in the presence of exogenous fatty acid may serve to promote expression of a positive acting transcription factor that is needed to activate farE. This would partially conform to the AcrR-AcrB paradigm, where AcrR ensures that acrB is not expressed in the absence of an inducing stimulus, but other positive acting factors are required to activate acrB (84-86). With these considerations in mind, work is in progress to determine the mechanism of FarR-dependent regulation of gene expression, through analysis of its interaction with different fatty acids and target promoters, and the scope of genes that are affected by this interaction.
2.5 References

1. Boucher H, Miller LG, Razonable RR. 2010. Serious infections caused by methicillin-resistant 


33. Nickerson N, Ip J, Passos DT, McGavin MJ. 2010. Comparison of Staphopain A (ScpA) and B (SspB) precursor activation mechanisms reveals unique secretion kinetics of proSspB (Staphopain B), and a different interaction with its cognate Staphostatin, SspC. Mol Microbiol 75:161–177.

34. Fey PD, Endres JL, Yajjala VK, Widhelm TJ, Boissy RJ, Bose JL, Bayles KW. 2013. A Genetic resource for rapid and comprehensive phenotype screening of nonessential *Staphylococcus aureus* genes. MBio 4:e00537-12-e00537-12.


61. Xiao Q, Vakulenko S, Chang M, Mobashery S. 2014. Mutations in mmpL and in


281:23129–23137.


Chapter 3

3 Identification of FarR binding sites responsible for auto-repression and efflux-mediated resistance to antimicrobial fatty acids in *Staphylococcus aureus*²

² This manuscript is in preparation.
3.1 Introduction

Methicillin-resistant *Staphylococcus aureus* has rapidly achieved pandemic status in community and hospital settings. To persist on human hosts, *S. aureus* must have intrinsic defense mechanisms to cope with antimicrobial unsaturated free fatty acids (uFFA), an important component of human innate defense mechanisms. In previous work, we identified a regulator of fatty acid resistance, FarR, which belongs to the TetR family of transcriptional regulators (TFR) (1). TFRs represent the third most ubiquitous transcriptional regulators in prokaryotes (2). This family is named after the prototypic TetR that has a role in mediating tetracycline resistance (3). Members of this family typically exert their effect on divergent genes in response to small hydrophobic ligands and are involved in the transcriptional regulation of a wide range of biological functions such as multidrug resistance, antibiotics biosynthesis, quorum sensing, and pathogenicity (4). Studies show that overexpression of multidrug efflux pumps, and the resulting antibiotic resistance, is often attributed to mutations in transcriptional regulators such as those belonging to the TetR family. In Gram-negative bacteria, AcrAB is an RND-efflux pump regulated by the TFR, AcrR, and other global transcriptional regulators such as MarA, SdiA, RobA and SoxS (5, 6). In this paradigm, the de-repression of AcrR in the presence of an inducing ligand is not enough to ensure that the pump is only expressed when needed, and additional positive-acting factors are needed to fully activate the efflux pump (7). As a result the TFR AcrR fine-tunes the expression of the efflux pump, as opposed to straightforward repression and de-repression (8). Nevertheless, mutations in *acrR* alone can cause overexpression of the AcrAB efflux pump and are linked to antibiotic resistance in clinical isolates (9). Similar to the *acrR* and *acrB* paradigm in *E. coli*, where *acrR* and *acrB* were discovered by in vitro selection of acriflavine-resistant mutants, we discovered *farR* through in vitro selection of USA300 variants with increased resistance to linoleic acid (1).

Analysis of the region upstream of *farR* revealed an RND efflux pump, *farE*, the expression of which is induced by antimicrobial uFFA, including linoleic and arachidonic acid. Importantly, a single nucleotide polymorphism causing a His_{121}Tyr substitution in FarR caused increased expression of *farE*, in the absence of an inducing stimulus, and a
significantly higher induced level of expression when cells are exposed to linoleic acid. Moreover, we showed that farE is required to support the growth of S. aureus in the presence of sub-inhibitory concentrations of linoleic acid, and that resistance to linoleic acid is proportional to farE expression (1).

The regulation of lipid homeostasis is essential to maintaining bacterial physiology and here we report the first steps towards understanding how FarR regulates the expression of farE. We previously established that farE is induced by antimicrobial fatty acids in a farR-dependent manner, providing evidence that farR does not function strictly as a repressor. TFRs are mainly repressors in nature, with limited reports of unconventional TFRs that have dual roles (10–12). SczA of Streptococcus pneumoniae, for instance, is an unconventional TFR that binds an operator site in the absence of its ligand, zinc, and binds another distinct operator site further upstream to act as an activator and help recruit the transcription machinery (13). We here examine FarR-DNA interaction and establish that FarR binds to distinct operator sites in the intergenic segment between the divergent farE and farR, and nucleotide mutation in these operator sites abrogated protein binding of FarR to these sites. Using a gene-reporter system, we determine that FarR, similar to the majority of TFRs, is auto-regulatory in nature and that the operator site responsible for auto-repression spans the -10 element of P_farR as well as +1 transcriptional start site of farR. Finally, we establish that FarR binds to a DNA operator site upstream of the putative farE promoter that may have a role in farE activation.

3.2 Materials and methods

3.2.1 Bacterial strains and growth conditions

A list of bacterial strains used in this study is provided in Table 3.1. Bacterial cultures were maintained as frozen stocks at -80°C in 20% glycerol and streaked on tryptic soy agar (TSA) plates (S. aureus) or Luria Bertani (LB) agar plates (E. coli), when needed. Antibiotics were added when needed at the following concentrations: chloramphenicol, 5 µg/ml; erythromycin, 5µg/mL; anhydrotetracycline, 250 ng/ml; ampicillin, 100 µg/mL and kanamycin, 50 µg/mL. For growth analyses, single colonies were inoculated into 3 mL TSB or LB broth supplemented with antibiotics, when required, and incubated
overnight at 37°C on an orbital shaker (VWR). Cultures were then diluted into 25 mL TSB to a starting OD$_{600}$ of 0.01 and incubated at 37°C on an orbital shaker at 180 rpm. For fatty acids supplementation, 25 mL TSB supplemented with 0.1% DMSO was used.

3.2.2 Construction of *S. aureus* USA300ΔfarER and USA300ΔfakA

In-frame, markerless deletion of both *farE* and *farR* as well as *fakA* was achieved using pKOR1 as previously described (14). Briefly, 1000 bp segments located upstream and downstream of the *farE-farR* locus were amplified using PCR primers that incorporate *attB1* and *attB2* sequences as well as a *SacII* cut site to allow for ligation of the resulting amplicons. The segment upstream of *farE* was amplified using *farE-UP-attB1* that incorporates *attB1* sequence on the 5′-end of the segment, and *farE-UP-SacII* that incorporates a *SacII* cut site on the 3′-end of the segment. The segment downstream of *farR* was amplified using *farR-DW-SacII* that incorporates a *SacII* site at the 5′-end of the segment, and *farR-DW-attB2*, which incorporates an *attB2* sequence on the 3′-end of the segment. Similarly, the *fakA* mutant was constructed by amplifying 1000 bp segments located upstream and downstream of *fakA* using PCR primers that incorporate *attB1* and *attB2* sequences as well as a *SacII* cut site to allow for ligation of the resulting amplicons. The amplicons were subsequently purified, digested with *SacII* and ligated together using T4 DNA ligase (NEB). The resulting fragment was then introduced into the pKOR1 vector using site-specific recombination. This was achieved using BP Clonase II (Invitrogen) to facilitate recombination between the *attB1* and *attB2* sequences on the ligated PCR product and the *attP1* and *attP2* sites on the vector, generating pKORΔfarER or pKORΔfakA. The vectors were constructed in *E. coli* DH5α background first and subsequently passaged through *S. aureus* RN4220 at 30°C before electroporation into *S. aureus* USA300. In-frame allelic replacement of *farER* or *fakA* was then achieved by a two-step temperature shift and anti-sense counter-selection as previously described (14). The USA300ΔfakA-ΔfarER double mutant was constructed by electroporating pKORΔfarER into *S. aureus* USA300ΔfakA, followed by in-frame allelic replacement as described above.
3.2.3 Construction of complementation and reporter gene constructs

Oligonucleotides used to generate reporter gene constructs, or to complement mutants can be found in Table 3.2. For complementation, we utilized the pLI\textit{farR} and pLI\textit{farE} complementation vectors that we previously made (1). \textit{farE} was cut out of pLI\textit{farE} using the restriction enzymes \textit{KpnI} and \textit{SacII}, and subsequently gel purified for ligation with pLI\textit{farR} in \textit{E. coli} DH5α background. The presence of ligated genes (\textit{farER}) was confirmed by PCR and DNA sequence analysis. The pLI\textit{farER} construct was then passaged through \textit{S. aureus} RN4220 and electroporated into USA300Δ\textit{farER}. The complementation construct pLI\textit{farR} was modified using the mutagenic primers \textit{P}\textsubscript{\textit{farR}}-10\textsubscript{G}→\textsubscript{A}-F and \textit{P}\textsubscript{\textit{farR}}-10\textsubscript{G}→\textsubscript{A}-R, using protocols and reagents following the QuikChange Site-Directed Mutagenesis Kit (Stratagene), to construct pLI\textit{farR}\textsubscript{TAG>A}, harboring a single nucleotide substitution within the -10 motif of the \textit{P}\textsubscript{\textit{farR}} promoter. To construct reporter gene constructs we utilized the pGY\textit{lux} vector where the luciferase operon can be driven from our promoter of interest (15). We previously constructed \textit{farE}::\textit{lux} by cloning a 397 bp fragment, which included the entire intergenic segment between \textit{farE} and \textit{farR} and additional sequence from the 5′-end of \textit{farE} and 5′-end of \textit{farR}, in front of the \textit{lux} operon (1). To localize the \textit{farR} promoter, we cloned two \textit{farR} segments into the pGY\textit{lux} vector; the larger segment (246 bp) that made up pGY\textit{farR}::\textit{lux}1 was amplified using primers pGY\textit{farR1}-F and pGY\textit{farR}-R, and the smaller segment (164 bp) that made up pGY\textit{farR}::\textit{lux}2 was amplified using primers pGY\textit{farR2}-F and pGY\textit{farR}-R. Both segments were cloned into the \textit{BamH}I and \textit{SalI} sites of pGY\textit{lux} (15). pLI\textit{farR}\textsubscript{TAG>A} was used as a template in PCR with primers pGY\textit{farR1}-F and pGY\textit{farR}-R, and the amplicon was then cloned in pGY\textit{lux} to generate pGY\textit{farR}\textsubscript{TAG>A}::\textit{lux}. 
<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus:</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA300LAC</td>
<td>Community-acquired methicillin-resistant <em>S. aureus</em>, wild-type strain cured of antibiotic resistance plasmids</td>
<td>(16)</td>
</tr>
<tr>
<td>RN4220</td>
<td>Restriction endonuclease deficient lab strain of <em>S. aureus</em> capable of accepting foreign DNA</td>
<td>(17)</td>
</tr>
<tr>
<td>USA300ΔfakA</td>
<td>USA300LAC with markerless deletion of <em>fakA</em> (SAUSA300_1119)</td>
<td>This study</td>
</tr>
<tr>
<td>USA300ΔfarER</td>
<td>USA300LAC with markerless deletion of <em>farE</em> (SAUSA300_2489) and <em>farR</em> (SAUSA300_2490)</td>
<td>This study</td>
</tr>
<tr>
<td>USA300ΔfakA-ΔfarER</td>
<td>USA300LAC with markerless deletion of <em>fakA</em>, <em>farE</em>, and <em>farR</em></td>
<td>This study</td>
</tr>
<tr>
<td>USA300ΔfarER(pLI50)</td>
<td>USA300ΔfarER with empty pLI50 vector, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>USA300ΔfarER(pLIfarE)</td>
<td>USA300ΔfarER complemented with native <em>farE</em>, cloned in pLI50, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>USA300ΔfarER(pLIfarR)</td>
<td>USA300ΔfarER complemented with native <em>farR</em>, cloned in pLI50, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>USA300ΔfarER(pLIfarR7)</td>
<td>USA300ΔfarER complemented with <em>farR</em> from SNP-containing clone FAR7, cloned in pLI50, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>USA300ΔfarER(pLIfarER)</td>
<td>USA300ΔfarER complemented with native <em>farER</em>, cloned in pLI50, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>λ&lt;sup&gt;+&lt;/sup&gt;φ80dlacZΔM15Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rK&lt;sup&gt;−&lt;/sup&gt; mK&lt;sup&gt;−&lt;/sup&gt;) supE44 thi-1 gyrA relA1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>M15[pREP]</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, φ80ΔlacM15, thi, lac&lt;sup&gt;−&lt;/sup&gt;, mtl&lt;sup&gt;−&lt;/sup&gt;, recA&lt;sup&gt;+&lt;/sup&gt;, KmR</td>
<td>Qiagen</td>
</tr>
<tr>
<td><strong>Plasmids:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pLI50</td>
<td><em>E. coli-S. aureus</em> shuttle vector</td>
<td>(18)</td>
</tr>
<tr>
<td>pLIfarE</td>
<td>pLI50 with native <em>farE</em> gene</td>
<td>(1)</td>
</tr>
<tr>
<td>pLIfarR</td>
<td>pLI50 with native <em>farR</em> gene</td>
<td>(1)</td>
</tr>
<tr>
<td>pLIfarR7</td>
<td>pLI50 with <em>farR</em> gene from the variant FAR7 clone</td>
<td>(1)</td>
</tr>
<tr>
<td>pLIfarR&lt;sub&gt;TAG&gt;A&lt;/sub&gt;</td>
<td>pLI50 with <em>farR</em> gene harboring a TAG&lt;sup&gt;A&lt;/sup&gt; mutation in the -10 element of P&lt;sub&gt;farR&lt;/sub&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>Vector Name</td>
<td>Description</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>pKOR1</td>
<td>E. coli-S. aureus shuttle vector containing P&lt;sub&gt;xyI-tetO&lt;/sub&gt; and expressing antisense secY RNA</td>
<td>(14)</td>
</tr>
<tr>
<td>pKORΔfarER</td>
<td>pKOR-1 containing upstream and downstream flanking sequences for deletion of farER</td>
<td>This study</td>
</tr>
<tr>
<td>pKORΔfakA</td>
<td>pKOR-1 containing upstream and downstream flanking sequences for deletion of fakA</td>
<td>This study</td>
</tr>
<tr>
<td>pGYlux</td>
<td>E. coli-S. aureus shuttle vector harboring promoterless luxABCDE operon</td>
<td>(15)</td>
</tr>
<tr>
<td>pGYfarE::lux</td>
<td>E. coli-S. aureus shuttle vector containing P&lt;sub&gt;farE&lt;/sub&gt; for luxABCDE operon</td>
<td>(1)</td>
</tr>
<tr>
<td>pGYfarR::lux1</td>
<td>E. coli-S. aureus shuttle vector containing P1 of farR for luxABCDE operon</td>
<td>This study</td>
</tr>
<tr>
<td>pGYfarR::lux2</td>
<td>E. coli-S. aureus shuttle vector containing P2 of farR for luxABCDE operon</td>
<td>This study</td>
</tr>
<tr>
<td>pGYfarR&lt;sub&gt;TAG-A&lt;/sub&gt;::lux</td>
<td>E. coli-S. aureus shuttle vector containing mutated P1 of farR for luxABCDE operon</td>
<td>This study</td>
</tr>
<tr>
<td>pQE30</td>
<td>E. coli vector for IPTG-inducible expression and incorporation of an N-terminal 6×His tag</td>
<td>Qiagen</td>
</tr>
<tr>
<td>pQE6His-farR</td>
<td>pQE30 containing 6×His tagged farR</td>
<td>This study</td>
</tr>
<tr>
<td>pQE6His-farR7</td>
<td>pQE30 containing 6×His tagged farR from the variant FAR7 clone</td>
<td>This study</td>
</tr>
<tr>
<td>pUC18</td>
<td>E. coli cloning vector</td>
<td>(19)</td>
</tr>
</tbody>
</table>
Table 3.2 Oligonucleotides used in Chapter 3.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>farR-DW-SacII&lt;sup&gt;a&lt;/sup&gt;</td>
<td>gccctcgcggGCGAAGATATTGATAAACATTTTCC</td>
</tr>
<tr>
<td>farR-DW-attB2</td>
<td>ggggacaactttgtacaaagagatggttGGTAATTTAGAACAAGGTCG</td>
</tr>
<tr>
<td>farE-UP-attB1</td>
<td>ggggacaagtgtgctacaaaaagagctgacctTTCCTTTGCTGATGTC</td>
</tr>
<tr>
<td>farE-UP-SacII&lt;sup&gt;b&lt;/sup&gt;</td>
<td>gccctcgcggACGATGCGGATTGTACCAAG</td>
</tr>
<tr>
<td>fakA-UP-attB1</td>
<td>ggggacaactttgtacaaagagctgacctGCGTGAGACGTTGCTGATTCCAGTCG</td>
</tr>
<tr>
<td>fakA-UP-SacII&lt;sup&gt;c&lt;/sup&gt;</td>
<td>gccctcgcggCATTTCAAGTTGTCCTCTTAAGCTTTTCTG</td>
</tr>
<tr>
<td>fakA-DW-attB2</td>
<td>ggggacaactttgtacaaagagctgacctGCGTGAGACGTTGCTGATTCCAGTCG</td>
</tr>
<tr>
<td>pGYfarE-F&lt;sup&gt;b&lt;/sup&gt;</td>
<td>cccggtgaccTTTAGTGACGAGTACGG</td>
</tr>
<tr>
<td>pGYfarE-R&lt;sup&gt;c&lt;/sup&gt;</td>
<td>cccggtgaccCGGTCGCATTTGAGACGTCG</td>
</tr>
<tr>
<td>pGYfarR1-F&lt;sup&gt;b&lt;/sup&gt;</td>
<td>cccggtgaccTGCAGCTACAATCATGACTACATGC</td>
</tr>
<tr>
<td>pGYfarR2-F&lt;sup&gt;b&lt;/sup&gt;</td>
<td>cccggtgaccGCCAACAGTTATGGCTCC</td>
</tr>
<tr>
<td>pGYfarR-R&lt;sup&gt;c&lt;/sup&gt;</td>
<td>cccggtgaccTAAATACGCTCTTCTTCACTCATTTCTCC</td>
</tr>
<tr>
<td>farR-GSP1</td>
<td>TTATCTGGAGGATGTCGTCG</td>
</tr>
<tr>
<td>farR-GSP2</td>
<td>CCGTCGACTCAGCGTCTCTTCTGG</td>
</tr>
<tr>
<td>farR-GSP3</td>
<td>ATTGTCGACACTCATCGTGGGAATGG</td>
</tr>
</tbody>
</table>
| AAP                   | GGCCACGCGTGCGACTAGTGCGGG/ideoxyI/GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
<table>
<thead>
<tr>
<th>OP5TAG&gt;A</th>
<th>AGTTAAATACGTTAATTATTGTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>OP5ΔTAG</td>
<td>AGTTAAATATACGTTATTATTGTT</td>
</tr>
<tr>
<td>OP6</td>
<td>AAATCAACGGTTATAGTTTTAATATA</td>
</tr>
<tr>
<td>OP7</td>
<td>TGTAGATTATTGTCGGATTATAGTATCT</td>
</tr>
<tr>
<td>OP11</td>
<td>GTATATTGCCCTCTTTTTAATCAACGTTA</td>
</tr>
<tr>
<td>OP12</td>
<td>GCCTCCTTTAAAAATCAACGTTATAGTTTA</td>
</tr>
<tr>
<td>OP13</td>
<td>TTTTAAAATCAACGTTATAGTTTTAATATA</td>
</tr>
<tr>
<td>OP13A</td>
<td>TAAAATCATTGTTATAGTTAAAT</td>
</tr>
<tr>
<td>OP13TAG&gt;A</td>
<td>TTTTAAAATCAACGTTATAGTTTTAATATA</td>
</tr>
<tr>
<td>P_{far}R-10G&gt;A-F</td>
<td>CTTTTAAAAATCAACGTTATAATTTAATATACAGTGAG</td>
</tr>
<tr>
<td>P_{far}R-10G&gt;A-R</td>
<td>CTACACTGTATATTTAAATTATAACGTTGATTTTAAGG</td>
</tr>
</tbody>
</table>

Lower case nucleotides are attB2 and attB1 site for cloning into pKOR1 vector. Lower case and bold nucleotides indicate the addition of 5’ sequences to incorporate restriction endonuclease cut sites as follows: aSacII, bBamHI, cSalI, dSacI, and eHindIII. Nucleotides in bold show the locations of site-directed mutations.
3.2.4 RNA isolation and 5’-rapid amplification of complementary DNA ends

RNA was isolated from *S. aureus* cells (USA300 and FAR7) grown to mid-exponential phase (OD$_{600}$ of 0.5) in TSB supplemented with 20 µM linoleic acid, using Aurum™ total RNA kit (Bio-Rad) as per manufacturer’s instructions. Synthesis of first-strand cDNA was prepared using 1 µM *farR*-gene specific primer *farR*-GSP1, 1.5 µg of RNA, 50 µM dNTPs, and 20 units of SuperScript™ II reverse transcriptase (Invitrogen), followed by incubation at 42°C for 50 minutes. The original mRNA template was subsequently removed by treatment with a mixture of RNaseH (0.5 units) and RNase T1 (50 units) at 37°C for 30 minutes. The cDNA was then purified using BioArray® cDNA purification kit (Enzo Life Sciences) as per manufacturer’s instructions to eliminate unincorporated *farR*-GSP1 and dNTPs. A dC-tail was added to the 3’-end of the purified cDNA using 20 units of terminal transferase (Roche) and 0.5 mM CTP, and the resulting C-tailed cDNA was utilized in a PCR reaction using abridged anchor primer (AAP) and nested *farR* gene specific primer *farR*-GSP2. A second PCR was then conducted, using the first PCR product as a template, with abridged universal amplification primer (AUAP) and a nested *farR*-GSP3 containing a SalI restriction cut site to allow for cloning into pUC18. Cloned products were sequenced using M13-F and M13-R primers that flank the multiple cloning site of pUC18. The +1 transcription start site was identified as the first nucleotide following the poly C- or G-tail, depending on the orientation of the cloned insert.

3.2.5 Construction and purification of recombinant FarR

*farR* genes from both USA300 and FAR7 were cloned in pQE30 to allow for expression with an N-terminal 6×His-tag using primers *farR*-6H-F and *farR*-6H-R. Recombinant proteins were expressed and purified from *E. coli* M15[pREP]. Bacterial cultures were grown at 37°C in LB (Sigma Aldrich) supplemented with 100 µg/mL ampicillin and 50 µg/mL kanamycin, to an OD$_{600}$ of 0.8 before the addition of 0.1 mM of isopropyl 1-thio-β-D-galactopyranoside (IPTG) and incubation at room temperature with shaking for additional 18 hours. Cells were harvested by centrifugation and resuspended in binding
buffer (20 mM sodium phosphate, 0.5 M NaCl, 40 mM imidazole, pH 7.4), lysed in a cell disruptor (Constans System Ltd.) at 25000 psi, and subsequently pelleted at 3000 rpm for 15 minutes. Supernatant was then ultracentrifuged for 50 minutes at 50,000 xg in a Beckman Coulter Optima L-900K ultracentrifuge, after which the soluble fraction was filtered through a 0.45µm Acrodisk® syringe filter (Pall Laboratory). The lysate was applied onto a 1 mL His-Trap nickel affinity column (GE Healthcare) that was equilibrated with binding buffer. After washing extensively with binding buffer, bound His-tagged protein was eluted over a linear imidazole gradient up to 0.5 M imidazole in 20 mM sodium phosphate. Column fractions were assessed by SDS-polyacrylamide gel electrophoresis to check for purity, and fractions were then pooled and dialyzed in 20 mM sodium phosphate, 0.5 M NaCl, pH 7.4 at 4°C overnight (Appendix 8). Protein concentration was determined by Bradford assay using Bio-Rad protein assay reagent.

3.2.6 FarR-DNA interaction studies

Electrophoretic mobility shift assays (EMSA) were performed using recombinant 6×His-tagged FarR and fluorescently-labeled duplex oligonucleotides probes. A list of oligonucleotides used in this study is provided in Table 3.2. IRDye-labeled single-stranded oligonucleotides were purchased from Integrated DNA technologies (IDT®) and complementary oligonucleotides were annealed at 100 µM each in 10 mM Tris, pH 8.0, 0.1 mM EDTA, by incubation at 95°C for 5 minutes followed by cooling down slowly to room temperature for 45 minutes. Each 25 µL EMSA reaction contained 5 pmol of fluorescently-labeled probe, up to 2 µM purified FarR, 240 µg/mL bovine serum albumin (BSA) and 15.2 µg/mL poly[d(I-C)] in 10% glycerol, 15 mM Tris-HCl pH 8.0, 0.5 mM MnCl₂, 60 mM KCl, 0.5 mM MgCl₂, and 8 mM dithiothreitol (DTT). Reaction mixtures were incubated at room temperature for 60 minutes, after which they were run on a 6% TBE-acrylamide gel for 45 minutes at 120V, and imaged using an Odyssey imager (LI-COR Biosciences). FarR-DNA interaction was determined as the conversion of unbound DNA probe to slow-moving protein-DNA complex. In competition assays, unlabelled competitor probes harboring specific nucleotide substitutions were added in 50-fold excess compared to that of the labelled probes.
3.2.7 Antibody production and western blotting

Rabbit polyclonal antisera recognizing FarR were generated by ProSci Incorporated (Poway, CA, USA). Two hundred µg of recombinant 6×His-FarR emulsified in complete Freund’s Adjuvant was used for the initial injection of rabbits, and 100 µg of the recombinant protein emulsified in incomplete Freund’s Adjuvant was subsequently used for each booster immunization. Rabbits were immunized every other week for a total of six weeks. For western blotting, cell lysates of *E. coli* complemented with pLI50 harboring native *farR* and *farR* bearing mutated *P_farR* were prepared by incubating washed cells with lysis buffer (150 mM NaCl, 5 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0, 1% (v/v) Triton X-100, 0.5% (v/v) SDS), supplemented with EDTA-free protease inhibitor cocktail (Roche), at room temperature for two hours with agitation, followed by centrifugation at 4,200 x g for 20 minutes. After determining protein content of the clarified cell lysate, samples containing 25 µg of total cell lysate protein were subjected to SDS-PAGE using a 12% polyacrylamide resolving gel. Proteins were then transferred to PVDF membrane following standard protocols. Primary anti-FarR antiserum was used at a dilution of 1:5000 followed by secondary IRDye800-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories Inc.). Membranes were imaged using Odyssey imager (LI-COR Biosciences).

3.2.8 Murine infection model

We utilized a skin abscess infection model as described by Malachowa et al. with adaptation (20). Briefly, female BALB/c mice were anesthetized by an isoflurane vaporizer, and flanks were treated with Nair hair removal lotion first, then shaved by a battery-operated trimmer one day prior to the inoculation to allow for proper visualization and measurement of abscesses. On infection day, bacterial cultures were inoculated at an OD₆₀₀ of 0.01 from an overnight stationary phase culture, and grown at 37°C with shaking to an OD of 2.0. Bacteria were harvested by centrifugation, washed twice with sterile PBS, and resuspended in sterile PBS to obtain a final concentration of 2x10⁸ cfu/mL. Bacterial suspensions were kept on ice until injection. Colony forming units in
each suspension were confirmed by plating on TSA and enumerating the next day. Mice were anesthetized by an isoflurane vaporizer, weighed, then challenged by subcutaneous injection of 50 µl of bacterial suspension (1x10⁷ cfu), or PBS only. Abscess progression was pictured daily to quantify abscess area by Image J. Animal weight was also monitored daily for three days, after which the animals were sacrificed, and the lesions were imaged. The infected lesions were then excised, homogenized in PBS-0.01% Triton X-100, and plated on Mannitol Salt Agar (MSA) for enumeration.

3.2.9 Computer analyses

Protein structural modeling was done using PHYRE2 (21). Analysis of DNA sequences and primer design were done using MacVector (Version 14.0.4 MacVector Inc.). Promoter predictions were performed using the Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/promoter.html), with adjustments for detection of prokaryotic promoters. Multiple sequence alignment was done using ClustalW 1.4 (22). Data points for growth, viability, and luciferase reporter gene assays were plotted and analyzed using GraphPad Prism, version 7.0. Statistical significance was determined using unpaired one-tailed Student’s t-test and two-way ANOVA using the statistical feature of GraphPad Prism, version 7.0.

3.3 Results

3.3.1 Deletion of farER results in loss of inducible resistance to linoleic acid

In our previous work, transposon insertions in farE and farR were used to demonstrate that the farE efflux pump, and the divergent transcriptional regulator farR were both required for inducible resistance of USA300 to the bactericidal activity of 100 µM linoleic acid (1). To confirm these observations, and to enable more detailed studies on gene expression and structure-function relationships USA300ΔfarER, where both genes are deleted, was constructed using pKOR1 markerless mutagenesis. In agreement with our previous observations, USA300ΔfarER suffered a loss of resistance to the bactericidal activity of 100 µM linoleic acid (Fig. 3.1A).
3.3.2  

*farR* is required for *farE* induction

The conventional mechanism of action of TFRs is to repress the expression of divergent genes. In previous work, we have shown that when assaying *farE::lux* reporter gene construct in USA300, the reporter activity was negligible in TSB only, but induced in TSB supplemented with 20 µM linoleic acid. We also observed that the reporter activity was negligible in USA300*farR::ΦNE* background suggesting that *farR* is required for induction of *farE* expression. Additionally, USA300Δ*farER* was unable to grow in TSB supplemented with 50 µM linoleic acid, and complementing with *farE* alone did not rescue growth, whereas complementing with pLI*farER* restored growth over 8 hours of incubation (Fig. 3.1B). As expected, when assaying *farE::lux* activity in USA300Δ*farER*, there was negligible induction of *farE* in the absence of *farR* function (Fig. 3.2). These data confirm that functional *farR* is indeed required for expression of *farE*. 
Figure 3.1 *farER* contributes to resistance of *S. aureus* USA300 to linoleic acid. A. Bactericidal assay: USA300, USA300ΔfarER, or USA300ΔfarER + pLIfarER were grown to mid-exponential phase in TSB + 20 μM linoleic acid, and then diluted to 10^6 cfu/mL in fresh TSB containing 100 μM linoleic acid. Viable cell counts were taken at hourly intervals. B. Growth assay: USA300ΔfarER complemented with pLIfarE, pLIfarER, or pLI50 vehicle was inoculated into TSB + 50 μM linoleic acid, and growth (OD$_{600}$) was monitored at hourly intervals. Each data point represents mean value of quadruplicate cultures. Error bars represent Standard Error of the Mean.
Figure 3.2 farR is required for farE induction. Growth (OD$_{600}$; open symbols) and relative luminescence units (RLU/OD; closed symbols) of USA300 and USA300ΔfarER harboring the pGYfarE::lux reporter construct are shown. Strains were grown in TSB or TSB supplemented with 20 µM linoleic acid (LA). Each value represents the mean and standard deviation of three separate cultures, and each culture was subjected to quadruplicate luminescence readings at each time point. Error bars represent Standard Error of the Mean.
3.3.3 Identification of \( \text{farR} \) promoter and transcription start site

The neighboring genes \( \text{farE} \) and \( \text{farR} \) are divergently transcribed. The intergenic segment between \( \text{farE} \) and \( \text{farR} \) translational start sites is 145 nucleotides, and bioinformatics analyses identified two potential \( \text{farR} \) promoters, P1 and P2, in this intergenic segment. P1 overlaps with the \( \text{farE} \) translational initiation codon on the minus strand, and is located 145 bp upstream of the translational initiation codon of \( \text{farR} \), whereas P2 is located 132 bp upstream of the translational initiation of \( \text{farR} \) (Fig. 3.3A). To confirm \( P_{\text{farR}} \) in the context of transcriptional initiation of \( \text{farR} \), we used 5′-RACE to determine the +1 site of \( \text{farR} \). The transcription start site of \( \text{farR} \) was located at 104 bp from the translation start site in two out of three independent experiments, and one experiment showed that the transcription start site was 105 bp from the gene’s start codon. These results correspond to P2 being the primary \( P_{\text{farR}} \) (Fig. 3.3A). To confirm this, we designed two overlapping \( P_{\text{farR}} \) segments to clone reporter gene fusions in pGYlux. The larger construct, \( \text{farR}::\text{lux1} \), harbors structural elements of both promoters P1 and P2 whereas the shorter truncated construct, \( \text{farR}::\text{lux2} \), lacks the -35 element of P1. When these reporter constructs were assayed in \( \text{E. coli} \), both displayed luminescence activity but the truncated \( \text{farR}::\text{lux2} \) exhibited 8-fold less activity compared to that of the larger \( \text{farR}::\text{lux1} \) construct (Fig. 3.3B). These data suggest that P2 is sufficient to promote transcription of the gene, although contribution of the overlapping P1 cannot be excluded. In a similar fashion, bioinformatics analyses identified a putative \( \text{farE} \) promoter in the \( \text{farER} \) intergenic segment that is located 102 bp upstream of the translational initiation codon of \( \text{farE} \); however, 5′-RACE experiments failed to determine the +1 transcription start site of \( \text{farE} \).

3.3.4 Expression of \( \text{farR} \) is subject to auto-regulation

Members of the TetR family of transcriptional regulators are typically subject to auto-regulation, including CamR in \( \text{Pseudomonas putida} \), TcmR in \( \text{Streptomyces glaucescens} \) and the well-characterized TetR. Conversely, QacR of \( \text{S. aureus} \) was not subject to auto-regulation (23). To determine whether \( \text{farR} \) is auto-regulated, we assayed \( \text{farR}::\text{lux1} \) activity in both USA300 and USA300\( \Delta\text{farER} \) backgrounds, and observed that the reporter activity was negligible in USA300, but was strongly de-repressed in
USA300ΔfarER. These data support farR auto-regulation such that farR::lux reporter activity is higher in the USA300ΔfarER background, due to the absence of functional FarR, concomitant with de-repression of PfarR. (Fig. 3.3C).
Figure 3.3 Nucleotide sequence of farER intergenic segment showing P_{farR} and P_{farE} promoter features (A), and farR::lux reporter gene assays conducted in *E. coli* (B) and *S. aureus* (C). The farER intergenic segment contains two potential P_{farR} promoters, P1 and P2, and a predicted P_{farE} promoter. The experimentally determined +1 transcription start site (TSS) of P2_{farR} was confirmed using three independent 5’- RACE experiments. Translation initiation sites of farE (TTG) and farR (ATG) are in gray boxes. RBS (ribosomal binding site) of farE and farR are shown. B. Assay of farR::lux promoter constructs in *E. coli*. The larger farR::lux1 construct contains both P1 and P2 promoters, while in farR::lux2, the P1 promoter is truncated within the predicted -35 promoter element. C. Assay of farR::lux1 in *S. aureus* USA300 and USA300ΔfarER. Each value represents the mean and standard deviation of three cultures, and each culture underwent quadruplicate luminescence readings at OD_{600} of approximately 0.1 (B), and 0.5 (C). Results are shown normalized to the optical density. Statistical significance is determined using unpaired one-tailed Student’s *t*-test, where *P < 0.05 and ****P < 0.0001. Error bars represent Standard Error of the Mean.
3.3.5 Identification of FarR operator sites

Although the divergent arrangement of the *farE*-far*R* genes matches the classical paradigm of TFR function, in which the TFR serves to repress the divergently transcribed gene, our data indicate that FarR is needed to express FarE, rather than functioning as a repressor. However, as with a number of other TFR’s, our data indicate that FarR represses its own expression. Therefore, we hypothesized that the *farER* intergenic segment should have two distinct operator sites to support binding of FarR; one to facilitate auto-repression of P_{farR}, and the other to function as an activator of P_{farE}. To identify potential operator sites, recombinant N-terminal 6×His-tagged FarR was expressed in *E. coli* and purified by metal affinity chromatography, for use in electrophoretic mobility shift assay (EMSA) experiments.

To determine if binding determinants are localized within the 145 bp *farER* intergenic segment, PCR with IRDye800 labelled primers was first employed to generate three ~160 bp probes that collectively encompass the entire intergenic segment, the 5′-end of *farR*, and the 5′-end of *farE* (OP\textsubscript{IN}, OP\textsubscript{R}, and OP\textsubscript{E}, respectively). Using EMSAs, 6×His-FarR bound only to probe OP\textsubscript{IN} spanning the intergenic segment between the two genes, causing a mobility shift. This binding exhibited two protein-DNA complexes indicating that there are multiple binding sites of FarR within the intergenic segment (Fig. 3.4B).

Since this confirmed that the primary FarR binding sites are contained entirely within the intergenic segment OP\textsubscript{IN}, we proceeded to identify potential operator sites within this segment. TFRs bind palindromic, and often repeated, DNA operator sequences. Based on Pustell DNA matrix analysis of the 145 nucleotide intergenic segment that separates the translational starts of *farE* and *farR*, we identified a 17 nucleotide pseudo-palindrome containing three mismatches (PAL1), flanked by imperfect 16 nucleotide direct repeats (IR1 and IR2). These features span the transcriptional start site of the putative *farE* promoter region on the minus strand, and that of *farR* on the plus strand, thus comprising a likely site for binding of the FarR regulatory protein (Fig. 3.4A).
To evaluate the ability of these features to support FarR binding, we conducted EMSAs with 43mer probes centered on the intergenic segment; FarR bound to probe OP1 that encompasses the pseudo-palindrome as well as IR1 and IR2. FarR also bound OP2, which encompasses 6 nucleotides of 5’-end of the pseudo-palindrome, IR1 and structural elements of P_farR as well as the +1 transcription start site of farR, but failed to bind to OP3 that contains 2 nucleotides of the 3’-end of the pseudo-palindrome, IR2 and structural elements of the putative P_farE as well as the +1 transcription start site of farE. Additionally, the 52mer probe OP4, that spans the -35 element and additional sequence upstream of the putative P_farE, also supported FarR binding (Fig. 3.4C). Interestingly, we identified a second pseudo-palindrome (PAL2) that bears similarity to PAL1 and is located in OP4 (Fig. 3.4A). PAL2 is a 16 nucleotide palindrome with four mismatches located 12 bp upstream of the -35 element of P_farE. Therefore, there appears to be a FarR binding site centered on the structural elements of P_farR, as well as another binding site upstream of the putative P_farE.

To further refine these binding sites, we designed 28mer probes centered on OP1 and OP2 (Fig. 3.4A). The probe OP5 is centered over the pseudo-palindrome, as well as 11 nucleotides of the 3’-end of IR1 and 8 nucleotides of the 5’-end of IR2. OP6 contains 9 nucleotides of the 5’-end of the pseudo-palindrome and IR1, whereas OP7 contains 8 nucleotides of the 3’-end of the pseudo-palindrome and IR2. FarR bound to OP5 and OP6, both of which span the +1 transcriptional start of farR, but did not exhibit any binding to OP7 (Fig. 3.4D).

The OP5 probe contains the entire PAL1, and to confirm specificity of protein-DNA interaction at this location, we employed competition EMSAs. In these assays, 50-fold excess of unlabeled competitor DNA is added to the reaction mix prior to incubation with the labeled probe. Specificity is confirmed by loss of binding to the labelled probe and thus lack of mobility shift. To this end, we designed a probe, OP5A, where the central GTGTAG sequence of the pseudo-palindrome was mutated to ACACCA and used it in a competition EMSA with the labelled OP5. Altering this central sequence of OP5 abolished the competition for FarR binding completely, confirming specificity of FarR binding to this operator site (Fig. 3.4E).
A.

```
CGATGAATGTCCCCATCTTATATAAAAATTTTGCCAAAGTATATTGCCTCCTTTTAAAATCAACGTTATAGTTTAAATATACAGTGTAGATTATTGTTCGATTATAGTATCTATCCCCGACCTCT

TAA

AGAATCAATTGGAAAATTTTGTATATTAAACTACACACAAAGGAGAAATGTAGATGAAAGAGACTGATTT

GCTACTTACAGGGGTAGAATATATTTTTAAAACGGTTTCATATAACGGAGGAAAATTTTAGTTGCAATATCAAATTTATATGTCACATCTAATAACAAGCTAATATCATAGATAGGGGCTGGAGA

ATT

TCTTAGTTAACCTTTTAAAACATATAATTTGATGTGTGTTTCCTCTTTACATCTACTTTCTCTGACTAAA
```

---

The diagram shows a genetic sequence with various transcription factors and expression sites labeled. The sequence includes several transcription factors labeled as OP1, OP2, OP3, etc., and expression sites labeled as P$_{farE}$ and P$_{farR}$.
B.

6XHis-FarR

OP_e  OP_IN  OP_R

Free Probe

C.

6XHis-FarR

FarR-Probe

Free Probe

OP1  OP2  OP3  OP4

D.

6XHis-FarR

FarR-Probe

Free Probe

OP5  OP6  OP7

E.

6XHis-FarR

FarR-Probe

Free Probe

OP5 Without a competitor  Unlabelled OP5  Unlabelled OP5A
Figure 3.4 FarR operator sites are located in the farER intergenic segment. A. 145-nucleotide intergenic segment separates the translational initiation of farE and farR. Pseudo-palindromes PAL1 and PAL2 are in gray boxes. Inverted repeats IR1 and IR2 are underlined. Core promoter elements are indicated. Transcription start sites are in bold. For initial EMSA experiments with 6×His-FarR, PCR was employed to generate 167 bp IRDye-labelled probes OP_{IN}, OP_{R}, and OP_{E} that span the entire intergenic segment, the 5'-end of farR, and 5'-end of farE, respectively. The overlapping 43mers OP1, OP2, and OP3, and the 52mer OP4 collectively span the entire intergenic segment. The 28mers OP5, OP6 and OP7 and the 30mers OP11, OP12 and OP13 were designed to further narrow down the operator site that supports FarR binding. B. FarR binds operator OP_{IN} spanning the farER intergenic segment. In each reaction, 5 pmol of operator DNA is incubated with 0, 0.5 and 2 µM protein. C. and D. FarR binds to OP1, OP2, OP4, OP5 and OP6 and failed to bind OP3 and OP7. Each reaction contained 5 pmol of DNA with or without 2 µM protein. E. FarR binds to OP5 which spans the +1 transcription start site of farR, and nucleotide substitutions in PAL1 of this probe abrogates FarR binding. Competition EMSA experiments were done by incubating FarR with excess (50X) of unlabelled competitor probe for 30 minutes prior to incubating with 5 pmol of labelled probe.
3.3.6 FarR binds OP<sub>Act</sub> and OP<sub>Rep</sub>

We have strong evidence that far<sup>R</sup> is required for far<sup>E</sup> induction (Fig. 3.2). Consequently, it appears that FarR does not function strictly as a transcriptional repressor, as is the case with most TFRs. There have been reports of two TFRs, LuxR in Vibrio harveyi and SczA in Streptococcus pneumoniae, that deviate from the norm of this family, where the protein functions as both repressor and activator depending on the presence or absence of a modulating ligand (13, 24, 25). This lead us to reason that FarR functions in a similar fashion where it binds a certain operator to function as a repressor and binds another operator site where it functions as an activator. Since we have evidence that FarR bound the operator OP4 located upstream of the putative P<sub>farE</sub>, and that no other sequences upstream of P<sub>farE</sub> even within the 5′-end of far<sup>R</sup> supported protein binding, we speculated that binding to this operator would be most likely to activate far<sup>E</sup>. Additionally, FarR is subject to auto-regulation, and since operator probes OP2, OP5 and OP6 all support FarR binding, span P<sub>farR</sub> and immediately downstream of P<sub>farE</sub>, we reasoned that binding to one of these operators would be to repress far<sup>R</sup>, and perhaps far<sup>E</sup>. To elucidate the minimal operator site that supports FarR binding for repression, we designed 30mer probes that span the structural features of P<sub>farR</sub>, which is contained within the larger OP2 probe. OP11 spans 30 bp from the 5′-end of OP2, including the -35 element of P<sub>farR</sub>. OP12 spans the center of OP2 and P<sub>farR</sub> including 4 bp of the -35 element and the entire -10 sequence of the promoter. OP13 contains 30 bp from the 3′-end of OP2, the -10 sequence of P<sub>farR</sub> as well as the transcriptional start site of far<sup>R</sup> (Fig. 3.4A). We performed competition EMSAs where 50-fold excess of these unlabelled competitor DNA probes are added prior to incubation with the labelled probe OP2. Both OP11 and OP12 were unable to compete with OP2, whereas OP13 competed effectively, and prevented protein binding to the labelled OP2, such that no mobility shift was observed (Fig. 3.5).
Figure 3.5 Competition EMSA with OP2 reveals that an operator site for binding of FarR is located within OP13. EMSA reactions contained 0, 0.5, or 2 µM 6×His-FarR, 250 pmol of unlabelled competitor, and 5 pmol of labelled OP2 probe. OP13 competes successfully with OP2 for binding of FarR, whereas OP11 and OP12 do not.
From these experiments, there appears to be two distinct operator sites for FarR binding in the intergenic segment between farE and farR. One operator is OP4, which contains sequences located immediately upstream of the putative P_{farE}. Since we established that farR does not function strictly as a repressor, we reasoned that this operator, hereafter termed OP_{Act}, is the site of FarR binding to enable activation of farE. Another distinct operator site spans the overlapping OP5 and OP13 probes. OP5 is centered over the PAL1 feature, which contains the +1 TSS of P_{farR} at its 5'-end, while its 3'-end is adjacent to the +1 TSS of P_{farE} on the minus strand. OP13 is centered on P_{farR}, and contains both the -10 promoter element and +1 TSS of P_{farR}. Since these two overlapping operator sequences span P_{farR} and transcriptional start site of farR, and immediately adjacent to that of farE, we reasoned that they represent repression operator site, OP_{Rep} (Fig. 3.6A).

3.3.7 OP_{Rep} contains the site of farR auto-repression and a His_{121}Tyr substitution in FarR causes relief of auto-repression

Since farR is auto-regulatory, we reasoned that one of overlapping operators making up OP_{Rep} is the site of FarR binding to enable auto-repression. In previous work, we described a fatty acid resistant clone FAR7, which harbored a single nucleotide polymorphism causing a His_{121}Tyr substitution in FarR. This clone exhibited increased constitutive levels of farE, even in the absence of fatty acid inducer, and displayed increased resistance to bactericidal concentrations of linoleic acid (1). When the reporter gene construct, farR::lux, was assayed in a FAR7 background we noticed a relief of the auto-repression when compared to wildtype USA300 (Fig. 3.6B). Therefore, it seems that the His_{121}Tyr substitution, although not located within the N-terminal DNA binding domain of FarR, still results in less effective auto-repression. As such, we examined the ability of recombinant 6×His-tagged FarR protein of the variant FAR7 clone, hereafter termed FarR7, in mobility shift assays parallel to those done with the wildtype FarR protein. Interestingly, FarR7 was unable to bind to OP2, to which native FarR was able to bind efficiently (Fig. 3.6C). OP2 spans P_{farR} and the +1 transcriptional start site of farR. To further define the operator sequence that supports differential binding of FarR and FarR7, we examined FarR7 binding to OP11, OP12 and OP13 that span the entire length of OP2. Strikingly, FarR7 did not exhibit any binding to OP13, and since FAR7
displayed a relief of auto-repression, these data suggest that OP13, which spans the -10 promoter element and +1 transcription start site of *farR*, also represents a site where FarR binds to mediate auto-repression (Fig. 3.6C).
B. \textit{farR::lux} Activity in \textit{S. aureus}

C.
Figure 3.6 FarR and the variant FarR7 differ in binding to OP2 and OP13, both of which span core promoter elements of \( P_{farR} \). A. Mapping the oligonucleotide probes relative to core promoter elements of \( P_{farR} \) and \( P_{farE} \). Overlapping OP5 and OP13 that support FarR binding are grouped as \( OP_{Rep} \). FarR also binds OP4 located upstream of \( P_{farE} \), representing a potential activation site, \( OP_{Act} \). B. \( farR::lux \) is de-repressed in the \( S. aureus \) FAR7 background. USA300 and FAR7 harboring the \( farR::lux1 \) reporter construct were grown in TSB, and samples were withdrawn at OD\(_{600}\) of approximately 0.5 for the determination of luciferase activity. Each data point represents the mean and standard deviation of three cultures, and each culture sample was subjected to quadruplicate luminescence readings at OD\(_{600}\). Statistical significance is determined using unpaired one-tailed Student’s \( t \)-test, where **\( P < 0.01 \). Error bars represent Standard Error of the Mean. C. Mobility shift assay showing that FarR7 does not exhibit any binding to OP2 or OP13. In each reaction, 5 pmol of operator DNA is incubated with up to 2 \( \mu M \) protein.
3.3.8 Specificity determinants of FarR binding

Aside from the sequence similarities between PAL1 and PAL2, the OP4, OP5 and OP13 probes that comprise OP_{Rep} and OP_{Act} appear to share no obvious sequence similarities. However, EMSA experiments confirmed that OP4, OP5 and OP13 can cross-compete for FarR binding and that nucleotide substitutions in any of them abrogated this competition completely. Interestingly, all three operators contained a TAG sequence central to each operator (Fig. 3.7A). To determine if this represents a specificity determinant for protein binding, we performed a competition EMSA with probes containing nucleotide substitutions (OP5TAG>A, OP13TAG>A) or deletion (OP5ΔTAG) in this sequence and found that these mutations eliminated FarR binding (Fig. 3.7B). Furthermore, analysis of multiple sequence alignment of 13 staphylococcal species that contain divergent farER revealed that FarR specificity determinants are conserved among these staphylococci. Importantly, the TAG nucleotides located in OP4, OP5, and OP13 are highly conserved among these species (Fig. 3.8).

Interestingly, in OP13, the TAG nucleotides are overlapping with the -10 element of P_{farR} and this operator is the only operator that exhibited differential binding of FarR and FarR7. Additionally, our reporter gene assays revealed that farR::lux is de-repressed in the FAR7 background, thus providing further evidence that OP13 is the site of FarR auto-regulation. To correlate these data with in vitro analyses, we introduced the same substitution in OP13 (OP13TAG>A where the -10 element of P_{farR} is mutated) into the existing reporter gene construct, farR::lux by site-directed mutagenesis. When we assayed the mutated farR_{TAG>A}::lux luciferase activity in USA300, there was a relief of auto-repression similar to that seen in the USA300ΔfarER and FAR7 backgrounds, confirming that OP13 is indeed the site of auto-repression of farR (Fig. 3.9A and B). We also prepared cell lysate fractions from E. coli complemented with pLI50 harboring native farR and farR bearing mutated P_{farR} grown in LB and showed by western blotting that mutating P_{farR} indeed relieves auto-repression, and promotes FarR expression (Fig. 3.9C).
A.

**OP4**

3’ TAAAGAGGAAACACACATCAAATTATATGTTTTAAAAGGTT 5’
5’ ATTTCTCCTTTGTGTG TAGTTTAATATACA AAATTTTCCAA 3’

**OP5**

5’ AGTTTTAATATACAGTG TAGATTATTGTT 3’
3’ TCAAATTTATATGTCACATCT AATAACAA 5’

**OP13**

5’ TTTTTAAAATCAACGT TAGGT T TAAATATA A 3’
3’ AAAATTTTAGTTGCAATAT CAAATTTTATAT 5’

B.

![Diagram with gel electrophoresis results for OP4, OP5, and OP13 with and without competitors and different probes.](image-url)
Figure 3.7 OP4, OP5 and OP13 cross-compete with each other and nucleotide substitutions obliterate this competition. A. Sequence similarities shared between OP4, OP5 and OP13. Arrows indicate the orientation of the similar sequence. The +1 TSS of \textit{farR} is labelled +1 in OP5 and OP13, and nucleotides comprising the -10 promoter element of P_{\textit{farR}} are italicized in OP13. B. OP4, OP5 and OP13 cross-compete with each other. Nucleotide substitutions in OP5 and OP13 (TAG>A) prevented this competition with OP4, confirming that this site is a specificity determinant for FarR binding. Each reaction contained 0, 0.5, and 2 µM FarR mixed with 250 pmol of unlabelled competitor and 5 pmol of labelled probe.
Figure 3.8 Multiple sequence alignment of the intergenic segment between farE and farR among *S. aureus* USA300 and 12 other staphylococcal species that contain divergent farER. The alignment was generated using ClustalW 1.4. Underlined sequences represent FarR binding motifs in OP13; OP5; and OP4, respectively. Bold and highlighted nucleotides indicate FarR favored nucleotides at positions 4, 5 and 6 of each binding motif.
A. \textit{farR::lux} Activity in USA300

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figureA.png}
\caption{\textit{farR::lux} Activity in USA300}
\end{figure}

B. \textit{farR::lux} Activity in different backgrounds

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figureB.png}
\caption{\textit{farR::lux} Activity in different backgrounds}
\end{figure}

C. E. coli +

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figureC.png}
\caption{E. coli +}
\end{figure}
Figure 3.9 Nucleotide substitution in the -10 element of PfarR causes a relief of auto-repression. A. Growth (OD$_{600}$; open symbols) and relative luminescence units (RLU/OD; closed symbols) of USA300 harbouring the pGYfarR::lux and pGYfarR$_{TAG>A}$::lux reporter construct are shown. B. Assay of farR::lux and farR$_{TAG>A}$::lux in different genetic backgrounds. *S. aureus* USA300 harboring either pGYfarR::lux, or pGYfarR$_{TAG>A}$::lux were grown in TSB to an OD$_{600}$ of approximately 0.5, and cultures were processed for determination of luciferase activity. For comparative purposes, assays were also conducted on cultures of *S. aureus* FAR7, and USA300ΔfarER harboring native pGYfarR::lux. Each value represents the mean and standard deviation of three cultures and each culture underwent quadruplicate luminescence readings at each time point (A) or at OD$_{600}$ of approximately 0.5 (B). Error bars represent Standard Error of the Mean. C. Western blot for detection of FarR protein in cell lysates of *E. coli* complemented with pLI50, pLIfarR or pLIfarR$_{TAG>A}$. Each lane contains 25 µg of total cell lysate protein, and the control lane contains 5 ng of recombinant 6×His-FarR.
3.3.9  *farER* and fatty acid detoxification

Since *S. aureus* is incapable of β-oxidation of fatty acids, the metabolic fate of exogenous unsaturated fatty acids is incorporation into the phospholipid component of the membrane (26). Parson *et al.* identified the fatty acid kinase system that is responsible for processing the exogenous fatty acids that enter the cell. FakB1 and FakB2 bind saturated and unsaturated fatty acids, respectively. FakA then phosphorylates the fatty acids and the resulting acyl-PO₄ is either extended by FASII machinery or directly incorporated into phospholipid (27). Importantly, TFRs are typically responsive to small hydrophobic ligands and crystallography studies of these regulators, including the fatty acid degradation regulator FadR of *Bacillus subtilis*, have revealed that endogenous fatty acid derivatives such as acyl-coAs are often co-crystallized with the protein from the *E. coli* host (28). We reasoned that phosphorylated fatty acids could represent the physiologic ligand that modulates FarR function and interaction with its operator sites. Therefore, we assayed the reporter gene construct, *farE::lux* in USA300ΔfakA, to examine *farE* expression when the ability to phosphorylate fatty acids is abrogated. Interestingly, USA300ΔfakA exhibited increased constitutive levels of *farE*, but expression could not be further induced by exogenous linoleic acid (Fig. 3.10). Further, this mutant was also significantly more resistant to killing by bactericidal concentrations of linoleic acid compared to wildtype USA300, and this phenotype is *farE*-dependent (Fig. 3.11).
Figure 3.10 *farE::lux* exhibits elevated constitutive levels of expression in the absence of fatty acid kinase, *fakA*. Growth (OD$_{600}$; open symbols) and relative luminescence units (RLU/OD; closed symbols) of USA300 and USA300$\Delta fakA$ harbouring the pGY$farE::lux$ reporter construct are shown. Strains were grown in TSB or TSB supplemented with 20 µM linoleic acid (LA). Each value represents the mean and standard deviation of three separate cultures, and each culture was subjected to quadruplicate luminescence readings at each time point. Error bars represent Standard Error of the Mean.
Figure 3.11 USA300ΔfakA exhibits enhanced resistance to linoleic acid, in a farE-dependent manner. A. Strains were grown in 50 µM linoleic acid. B. Strains were grown to mid-exponential phase in 20 µM linoleic acid, and then diluted to $10^6$ cfu/mL in fresh TSB containing 100 µM linoleic acid. Viable cell counts were taken at hourly intervals. Each data point represents mean value of triplicate (A) or quadruplicate (B) cultures. Error bars represent Standard Error of the Mean.
3.3.10  *farER* influence virulence in a subcutaneous abscess infection model

Studies on staphylococcal murine abscesses reveal that antibacterial activity dwells in the free fatty acid fraction of the abscesses (29). NorD, a *S. aureus* multidrug efflux pump from the major facilitator superfamily, was upregulated during infection and played a part in the bacterial fitness in an infection abscess model (30). Similarly, NorB in the *S. aureus* MW2 strain bestows fitness advantages in the murine subcutaneous abscess model. The pattern of expression of nor and *tet38* pumps in murine abscess models was found to be distinct from that observed during *in vitro* cell culture, and this was reasoned to be due to environmental triggers that dictate the cellular response (31). The fatty acid kinase, *fakA*, has also been linked to virulence in a murine model of *S. aureus* dermonecrosis; *fakA*-deficient mutants were more pathogenic than wildtype and formed larger abscesses (32). To assess the contribution of *farER*-mediated fatty acid resistance to virulence *in vivo*, and link it to the hyper-virulent phenotype observed in a *fakA*-deficient mutant, we constructed a USA300Δ*fakA*Δ*farER* double mutant and utilized it in a murine abscess infection model. In agreement with a previous report, USA300Δ*fakA* exhibited a trend towards increased surface abscess area compared to wild type USA300, but this enhanced virulence phenotype was abrogated in USA300Δ*fakA*Δ*farER* (Fig. 3.12). In contrast, the single mutant, USA300Δ*farER*, showed no difference in virulence compared to that of wildtype USA300 (Appendix 7). Therefore, the increased basal level of *farE* expression in USA300Δ*fakA* appears to promote increased resistance to antimicrobial fatty acids *in vitro*, and enhanced virulence in a subcutaneous abscess infection model.
Figure 3.12 USA300ΔfakA-farER exhibits reduced virulence in a murine skin abscess model of infection. 6-week female BALB/c mice were infected with 2x10^7 CFU of USA300, USA300ΔfarER, USA300ΔfakA and USA300ΔfakA-farER. Assesses were imaged daily and surface area was quantified using ImageJ. A. Abscess surface area at 2 days post-infection. B. The virulence exhibited by USA300ΔfakA was in a farER-dependent manner consistently throughout the course of infection. The reduction in virulence exhibited by USA300ΔfakA-farER is significant relative to that of USA300ΔfakA as determined by a two-way ANOVA where *P < 0.05. Error bars represent Standard Error of the Mean. n=8 abscesses per strain.
3.4 Discussion

This work provides the first mechanistic insight into the regulation of the RND family efflux pump FarE, by the TFR FarR, which together function to confer resistance of *S. aureus* to antimicrobial unsaturated free fatty acids. FarR, as with other TFRs, is auto-regulatory in nature. The transcription of *farE* increases in response to linoleic and arachidonic acids and gene fusions demonstrated that expression of *farE* is induced only in the presence of functional FarR. This represents one of the very few TFRs with dual functionality. 6×His-tagged FarR binds an operator overlapping the -10 element of P_{farR}, upstream of the transcriptional start site of the gene, as well as another operator downstream of the transcriptional start site. 6×His-tagged FarR also binds a third operator upstream of P_{farE}. Taken together, our mobility shift and reporter-gene assays establish a working model for us to further refine the mechanism by which *farE* is regulated by *farR*. First, FarR binds to an operator site within OP13, which includes the -10 motif of P_{farR}, leading to strong repression of FarR expression. An overlapping but independent operator site within OP5 could potentially function to repress both *farR* and *farE*, while binding to a third site in OP4 which is upstream of the predicted P_{farE}, is most likely to promote activation of *farE* expression. For simplicity, we refer to OP13 and OP5 as OP_{Rep} and OP4 as OP_{Act}.

Reporter-gene assays show that FarR has a repressive effect on its own gene, independent of the addition of fatty acid ligand, as evident from the low activity of *farR::lux* during growth of USA300 in TSB. Consequently, during growth in TSB, our data indicate that *farR* is strongly auto-repressed. Accordingly, the introduction of a single base pair substitution in the -10 element of P_{farR} led to abolishment of FarR binding in mobility shift experiments, and relief of auto-repression in reporter-gene assays. Similarly, a single nucleotide polymorphism that resulted in a His_{121}Tyr substitution in FarR displayed a constitutive level of *farE* expression in the absence of a fatty acid inducer, concomitant with increased resistance to linoleic acid, and less effective repression of *farR*. Therefore, to identify the operator site responsible for auto-regulation, we looked for differential binding between FarR and the FarR7 protein. Although OP5 partially overlaps with OP13, it supported FarR7 binding, whereas OP13 did not, suggesting that the latter is the
dedicated site for auto-repression. Although the location of this His\textsubscript{12}Tyr substitution was not within the N-terminal DNA binding domain of FarR, our data suggest that it does have a role in maintaining DNA-binding affinity. In a similar example, certain residues of the C-terminal ligand domain of \textit{B. subtilis} FadR undergo structural changes upon acyl-CoA binding, to affect the conformation of the DNA-binding domain. Mutations in these residues resulted in lower DNA binding affinity (28).

Since 6\times\text{His}-tagged FarR also binds OP5, it is feasible that OP5 is also a site for \textit{farE} repression. Typical repressors such as the prototypic TetR bind inverted repeats located downstream or spanning the -10 element of their respective promoters (28), and OP5 is located immediately downstream from the putative +1 transcription start site of \textit{farE}. Although binding to OP5 may not prevent RNA polymerase binding as anticipated of typical transcriptional repressors, it would still obstruct transition of the transcription complex, causing repression. Similarly, the staphylococcal QacR, a repressor of multidrug efflux pump QacA, binds an inverted repeat located immediately downstream of the \textit{qacA} promoter. This operator also overlaps the transcription start site of \textit{qacA} to block the transition of the RNA polymerase-promoter complex that otherwise allows for gene transcription (34, 35). Additionally, RutR, a TFR in \textit{E. coli}, represses its own expression by binding to an operator site located downstream of its transcription start site. This binding mode seems to be indicative of negative regulation by either interfering with open complex formation, elongation complex initiation, or RNA polymerase transition (36, 37). Conversely, both FarR and FarR7 bind OP4, located upstream of \textit{P}_{\text{farE}}, equally well, and do not bind any other operators upstream of \textit{P}_{\text{farE}} or within the 5’-end of the divergent \textit{farR}, suggesting that binding to OP4 would be most likely to activate \textit{farE}.

Studies show that the activation of the \textit{acrAB} promoter in \textit{E. coli} is in response to accumulation of cellular metabolites, and that global transcriptional activators (MarA and SoxS) and a local TetR-transcriptional repressor (AcrR) regulate the RND-efflux pump AcrB, to ensure that this pump is only expressed when needed. Binding of a wide range of toxic compounds to the C-terminal ligand binding domain of AcrR results in a conformational change in its N-terminal DNA binding domain, and this in turn releases the protein from operator DNA and permits transcription. However, the inactivation of
AcrR must also be accompanied by the induction of global regulators in order to achieve acrAB expression (8, 7, 38). Consequently, relief of de-repression alone is insufficient to promote constitutive expression of the AcrB efflux pump. In the context of FarE and FarR, binding of FarR to both OP\textsubscript{Rep} and OP\textsubscript{Act} may be a regulatory mechanism by which FarR controls the expression of the farE efflux pump to ensure it is only expressed when needed. We also examined all three operators that supported FarR binding and attempted to identify any common sequence that may be a specificity determinant for protein binding. There seems to be a clear preference for a common TAG(T/A)TTA binding motif in all three operators; 5’GTG\textsubscript{TAG}TTAAT3’ in OP4, 5’GTG\textsubscript{TAG}ATTATT3’ in OP5, and 5’T\textsubscript{TAG}TTTAAA3’ in OP13. We performed competition EMSAs with a combination of mutations and deletions of these motifs and found that FarR discriminates in favor of TAG nucleotides at positions 4, 5 and 6 of each binding motif. Interestingly, analysis of multiple sequence alignment of 13 staphylococcal species that contain divergent farER revealed that FarR binding motifs are conserved among these staphylococci. Importantly, the TAG nucleotides located in the binding motifs of OP4, OP5, and OP13 are highly conserved among these species (Fig. 3.8).

Structural studies will be needed to further shed light on the exact regulatory mechanism of FarR and its mechanism of de-repression and farE activation. There have been reports of TFRs with unconventional regulatory functions, such as RutR, that can both activate and repress gene expression in its apo-form. RutR is auto-regulatory, represses a divergently transcribed gene, and activates yet a third promoter. The mechanism by which RutR activates gene expression is yet to be determined, but the location of its operator sites suggests a mechanism of de-repression rather than actual recruitment of transcription machinery (36, 39). Another unconventional TFR is AmtR of Mycobacterium\textsubscript{smegmatis} which is involved in a post transcriptional regulation of urea metabolism without binding to any effector molecule. This regulation utilizes a sensory system that involves a trans-acting sRNA in response to nitrogen availability. When nitrogen is overabundant, the sRNA targets amtR mRNA and blocks its translation. Under nitrogen starvation, the sRNA is down-regulated and amtR is successfully translated. The AmtR repressor then works in combination with the global nitrogen
activator, GlnR, to fine-tune the expression of urea-degrading metabolic pathway (40). Another possibility is that FarR exhibits a regulatory network similar to that of DhaS, an unconventional TFR from *Lactococcus lactis* that functions as an activator. Similar to FarR, DhaS binds an operator site upstream of the Dha promoter, spanning the -35 element of the promoter. The mode of action of DhaS is not a direct de-repression, but rather involves a transcription co-activator, DhaQ, that forms a complex with DhaS upon ligand, dihydroxyacetone, binding and subsequently activates transcription of the dihydroxyacetone operon (12).

Upon entry into the cell, exogenous fatty acids bind FakB, and after phosphorylation by FakA, the resulting acyl-PO$_4$ is incorporated into phospholipid directly, or alternately, the PO$_4$ is exchanged for a CoA moiety, and the fatty acid passes through an extension cycle by the FASII machinery. A recent report by Lopez *et al.* has demonstrated that a Type VII secretion system (T7SS), which is crucial for prolonged bacterial survival and persistence of *S. aureus* in abscesses, is activated by uFFAs such as linoleic and arachidonic acids. Additionally, this activation is contingent on the incorporation of uFFAs into phospholipids and lipoprotein by the Fak machinery (41, 42). Therefore, the similar virulence we observe in wildtype USA300 and USA300ΔfarER is perhaps due to functional Fak machinery. Despite the evidence of a connection between farER and the fatty acid kinase *fakA* *in vitro* and *in vivo*, a direct interaction between FarE, FarR and FakA has yet to be demonstrated. Here we show that USA300ΔfakA exhibits increased constitutive levels of *farE*, but expression cannot be further induced by exogenous linoleic acid. This mutant was also significantly more resistant to killing by bactericidal concentrations of linoleic acid compared to wildtype USA300, and this phenotype was *farE*-dependent. A recent report indicates that USA300ΔfakA exhibited an elevated pool of intracellular fatty acids (43). Consequently, as reported in other RND efflux systems, an increased pool of intracellular metabolites in USA300ΔfakA may be responsible for increased basal expression of *farE*. Interestingly, FarR binding to OP$_{Rep}$ was partially reduced in the presence of linoleoyl-CoA and arachidonoyl-CoA (Fig. 3.13). Therefore, perhaps the accumulation of intracellular fatty acids results in the *fakA* mutant background leads to partial de-repression of *farE*, which cannot be fully induced due to
the fatty acids being unable to be phosphorylated in the absence of \textit{fakA}. As such, it appears likely that phosphorylated fatty acids are the signal for regulation of \textit{farE} via \textit{farR}. Experiments to test this would contribute to our understanding of the complex \textit{farR}-dependent activation of \textit{farE}. Antimicrobial unsaturated fatty acids represent a vital component of innate immunity, and \textit{S. aureus} is constantly exposed to them, not only during colonization but also in the context of an infection. It is evident that one of the strategies \textit{S. aureus} employs to cope with these fatty acids is via a complex regulatory mechanism where a TFR with dual functionality controls the expression of a fatty acid specific efflux pump.
Figure 3.13 FarR binding to OP<sub>Rep</sub> is partially affected by linoleoyl-CoA and arachidonoyl-CoA. In each EMSA reaction, 5 pmol of DNA is incubated with 0, 0.5 or 2 µM FarR in the presence or absence of 50 µM ligand, as indicated.
3.5 References


Chapter 4

4 General Discussion and Conclusions
4.1 Summary

*S. aureus* USA300 is the current epidemic strain of CA-MRSA in North America. It accounts for 98% of MRSA infections presented to emergency departments in the United States. This strain is notorious for its ability to overproduce toxins and virulence factors, as well as its remarkable ability to persist on skin surfaces. In order to persist on skin surfaces, the bacterium must have intrinsic mechanisms to cope with the antimicrobial uFFAs that constitutes an important component of the immune defense mechanisms on the skin. These uFFAs are membrane-disruptive, and humans deficient in the production of these fatty acids are more susceptible to *S. aureus* infections. The purpose of this thesis was to better understand the genetic basis for *S. aureus* USA300 adaptation to uFFAs which allows this strain to establish a successful infection. When USA300 variants were selected for their ability to grow in elevated levels of uFFAs, two out of the seven clones that were sent for whole genome sequencing contained a SNP in an uncharacterized gene, *SAUSA300_2490*. Using domain enhanced lookup time accelerated BLAST, the amino acid sequence of *SAUSA300_2490* has 99% homology to TFRs. Furthermore, structure prediction using homology modeling server, Phyre2, predicts that *SAUSA300_2490* shares 99.8% amino acid sequence similarity with FadR, a TFR of fatty acid degradation in *T. thermophilus*. Typically, TFRs exert their effects on divergent genes. Bioinformatics revealed that the *SAUSA300_2489* gene, which is divergently transcribed from *SAUSA300_2490*, encodes a gene product that belongs to the Resistance-Nodulation-Cell Division (RND) superfamily of proteins, which promote proton-antiport dependent efflux mechanisms. The *SAUSA300_2489* gene has an MMPL domain that codes for members of putative integral transmembrane proteins that are linked with lipid-metabolizing enzymes, suggesting a possible role in the efflux of fatty acids. As such, in chapter 2 my aim was to characterize *SAUSA300_2490* and its divergent gene, their role in fatty acid resistance, and also characterize the effect of the SNP that lead to the identification of these two genes. First, I show that resistance to antimicrobial uFFAs is inducible in *S. aureus*. Second, *SAUSA300_2490* which I named *farR*, for a regulator of fatty acid resistance, is required for the inducible resistance to uFFAs. Third, *SAUSA300_2489* which I named *farE*, for an effector of fatty acid resistance, is a contributor to *S. aureus* persistence in the presence of antimicrobial uFFAs. Importantly, this *farE*-dependent
persistence is not only in the hyper-virulent MRSA, but also in methicillin-susceptible (MSSA) background. Furthermore, I show that FarE is an efflux pump in which its inactivation results in elevated uptake of [\textsuperscript{12}C]linoleic acid into bacterial cells. Fourth, I show that a SNP that resulted in His\textsubscript{121}Tyr substitution in the regulator FarR is alone sufficient to promote increased resistance to bactericidal levels of uFFAs. Unexpectedly, the growth analyses and reporter-gene assays of this chapter show that FarR is required for farE expression; a phenotype unusual for a typical TFRs. To follow up on this phenotype, I constructed an in-frame, markerless deletion of both farR and farE to conduct further studies on gene expression and structure-function relationships as I utilized transposon insertions mutants in all analyses of chapter 2.

In chapter 3, my aim was to characterize FarR, its regulatory function and its operator sites. First, using USA300ΔfarER I show that farE cannot be induced in the absence of FarR, thus confirming that FarR is a TetR family activator. Second, I show that, unlike the staphylococcal QacR, FarR is auto-regulatory by binding to an operator site spanning its transcription start site. Third, I utilize electrophoretic mobility shift assays to examine the binding of His-tagged recombinant FarR to DNA operators with varying lengths and locations. I show that FarR utilizes three operator sites located in the farER intergenic segment to exert its regulatory functions. One operator spans the -10 core promoter element of P\textsubscript{farR} as well as its transcription start site, and represents the site of auto-repression of farR. Another operator that is located immediately downstream of the predicted transcription start site of farE, and represents a potential site of repression of both farR and farE. A third operator is located upstream of P\textsubscript{farE}, and represents the potential site of activation of farE. Interestingly, I also show that although the His\textsubscript{121}Tyr substitution in FarR is not physically located within its N-terminal DNA binding domain, it still affects DNA binding affinity and causes a relief to the auto-regulation. I also examine whether the FarR-dependent regulation of the fatty acid efflux pump FarE is linked to the mechanisms of fatty acid detoxification in S. aureus. It is established in the literature that fatty acid kinase deficiency in S. aureus promotes hyper-virulence in murine models of skin infection. I also show that this virulence is farE-dependent in vitro and in vivo, and that fatty acid kinase deficient mutants exhibit elevated constitutive
levels of farE that cannot be induced by exogenous fatty acids; an intriguing phenotype that contributes to what is established in the literature regarding S. aureus pathogenesis.

4.2 Limitations and future studies

FarR is a novel TFR that functions as a repressor in the absence of a fatty acid inducer, while acting as an activator in its presence. The transcription of farE increases in response to linoleic and arachidonic acids, and gene fusions demonstrated that expression of farE is induced only in the presence of functional FarR. Yet mobility shift assays show that FarR binds with similar affinities to OPRep and OPAct. These findings raise two questions that my studies could not address; first, what is the mechanism by which FarR activates farE? And second, what is the ligand mediating this activation?

There are very limited reports of TFRs that exert positive-regulatory roles. DhaS, for example, is an unconventional TFR that functions as an activator (1). It binds an operator site upstream of the dha promoter, not to directly de-repress its target gene, but rather to bind a transcriptional co-activator, DhaQ. DhaQ then forms a complex with DhaS and subsequently activates transcription. In this situation, it is the transcriptional co-activator DhaQ which binds the physiologic ligand, dihydroxyacetone. Consequently, the TFR DhaS will only form a complex with the ligand-bound form of the transcriptional co-activator DhaQ (1). In consideration of this example, it is possible that FarR requires a co-activator that is yet to be determined; potentially either FakA and/or FakB2.

Perhaps identification of the endogenous ligand of FarR can aid in determining the exact mode of its regulation. TFRs typically bind hydrophobic ligands. Crystallography studies of FadR of B. subtilis, for example, show that acyl-CoAs are often co-crystallized with the protein from the E. coli host (2). Furthermore, FarR binding to OPRep is partially reduced in the presence of linoleoyl-CoA and arachidonoyl-CoA; however, it cannot be concluded that acyl-CoAs are the endogenous ligand for FarR. It is most likely that phosphorylated fatty acids are the ligand modulating the binding of FarR to its operator sites. A fakA-deficient mutant exhibit elevated constitutive levels of farE that cannot be further induced, due to the fact that fatty acids cannot be phosphorylated in the absence of this kinase. Purification of the fatty acid kinase proteins and the subsequent manual
phosphorylation of fatty acids for the use in mobility shift assays can help in confirming that phosphorylated fatty acids are the signal for regulation of farE via farR. Additionally, work is in progress to express FarR with a C-terminal 6×His tag in S. aureus. With this reagent in place, we could purify 6His-FarR from lysates of S. aureus grown in presence or absence of linoleic acid, and then conduct mass spectrometry to identify any protein or small molecule ligands that are captured in complex with FarR.

S. aureus can only synthesize branched- and straight-chain fatty acids as its genome does not code for any membrane phospholipid desaturase for synthesis of unsaturated fatty acids (3). Instead, the bacterium employs the fatty acid kinase (Fak) machinery to utilize host-derived uFFA for incorporation into the otherwise energetically expensive membrane lipid components (phospholipids, lipoproteins and cardiolipin) as well as lipoteichoic acids (4–6). A recent report by Lopez et al. has demonstrated that a Type VII secretion system (T7SS), which is crucial for prolonged bacterial survival and persistence in abscesses, is activated by uFFAs such as linoleic and arachidonic acids (7). Additionally, this activation is contingent on the incorporation of uFFAs into phospholipids and lipoprotein by the Fak machinery. This incorporation and subsequent stimulation of T7SS are required for virulence of S. aureus in a murine bacteremia model (7). Our data also provide evidence of a connection between the efflux pump FarE and fatty acid kinase FakA in vitro and in vivo. Since exogenous uFFA utilization by Fak machinery is central for S. aureus virulence, tight transcriptional regulation is then required to permit incorporation of uFFA into phospholipids, and induction of farE only when the metabolic capacity for incorporation into phospholipid is exceeded. Therefore, it is beneficial for the bacterium to fine tune the expression of farE such that it is induced rapidly when the cellular threshold for uFFA incorporation into phospholipid is exceeded, and subsequently represses the pump’s expression once the fatty acid concentration falls below that threshold. This may explain the complex FarR-mediated regulatory mechanism of FarE, to ensure that the efflux pump is only expressed when needed. This also may explain the observation that FarR binds with similar affinities to OPRep and OPAct as well as both FarR and the variant FarR7 bind equally well to OPAct. Perhaps FarR binds constitutively to OPAct but the binding to OPRep is what modulates the tight
regulation of farE expression. Therefore, in the absence of inducing ligand, binding to OP_{Rep} can repress farE and in the presence of inducing ligand, binding to OP_{Rep} is reduced, and binding to OP_{Act} can then contribute to activation of farE (Fig. 4.1). Further, analysis of the putative farE promoter reveals a GC-rich spacer between the predicted -10 and -35 promoter elements. This spacer, as opposed to a more typical AT-rich spacer in strong promoters, is indicative of reduced promoter activity that requires a transcriptional activator to help recruit RNA polymerase and the transcription machinery (8), further supporting the requirement for FarR as an activator of farE.

Reporter-gene assays show that FarR auto-represses its own expression, independent of the addition of fatty acid ligand. This raises the question as to why farR expression cannot be de-repressed in the presence of fatty acid inducer, while farE expression is de-repressed. Perhaps FarR does not directly require ligand binding, but rather employs a more complex regulatory mechanism that senses the fatty acid pool in the cell. The TFR AmtR, for instance, is involved in a post-transcriptional regulation of urea metabolism without binding to any effector molecule (9). This regulation utilizes a sensory system that involves a trans-acting sRNA in response to nitrogen availability. When nitrogen is overabundant, the sRNA targets amtR mRNA and blocks its translation. Under nitrogen starvation the sRNA is down-regulated and amtR is successfully translated. The AmtR repressor then works in combination with the global nitrogen activator, GlnR, to fine-tune the expression of urea-degrading metabolic pathway (9). Additionally, LuxR of V. harveyi that deviates from the norm of TFRs and functions as both repressor and activator depending on the presence or absence of a modulating ligand, is also subject to sRNA-mediated regulation to ensure tight post-transcriptional control of quorum sensing genes (10). Small RNAs can be encoded on the opposite strand of target mRNA, and despite the complementarity, they block translation. These antisense RNA molecules often target 5′ untranslated regions (UTR) that are close to or overlapping the ribosomal binding site of target genes (11–13). Therefore, it is possible that FarR employs tight post-transcriptional regulatory mechanism to ensure that the efflux pump is only expressed when needed. Not only the spatial arrangement of P_{farR} and P_{farE} on the plus and the minus strands conform to the typical antisense and target mRNA paradigm, but also analysis of putative P_{farE} did
reveal a 5'-UTR, upstream of the ribosomal binding site of farE, that is indicative of post-transcriptional regulation (Fig. 4.2). Further, analysis of P_{farR} revealed an even longer 5'-UTR, compared to that of farE, that also suggests a possible post-transcriptional regulation of farR (Fig. 4.2). Structural and functional studies will be needed to further shed light on the exact regulatory mechanism of FarR and its mechanism of de-repression and farE activation.

In conclusion, this thesis reports the first description of a specific mechanism of inducible resistance to antimicrobial fatty acids in a Gram-positive pathogen, and the first staphylococcal TFR that functions as an activator and a repressor. It is evident that one of the strategies S. aureus employs to cope with antimicrobial fatty acids and persist successfully on a human host is via a complex regulatory mechanism where a TFR with dual functionality controls the expression of a fatty acid specific-efflux pump.
Figure 4.1 Schematic model of the potential mechanism of $P_{farE}$ regulation by FarR, depending on exogenous fatty acid ligand. FarR binds constitutively to $OP_{Act}$ but the binding to $OP_{Rep}$ is what modulates the regulation of $farE$ expression. A. In the absence of inducing ligand, FarR binds to $OP_{Rep}$, thereby blocking transcription from $P_{farE}$. B. In the presence of inducing ligand, binding to $OP_{Rep}$ is reduced, and binding to $OP_{Act}$ can then lead to activation of $farE$. 
End of complementarity to farR transcript

farE start codon (TTG)

farE +1 TSS

5’

3’

Green: Stems (canonical helices)
Red: Multiloops (junctions)
Yellow: Interior Loops
Blue: Hairpin loops
Orange: 5’ and 3’ unpaired region
Green: Stems (canonical helices)
Red: Multiloops (junctions)
Yellow: Interior Loops
Blue: Hairpin loops
Orange: 5' and 3' unpaired region
Figure 4.2 Secondary structure prediction of *farE* and *farR* RNA using RNAfold Webserver. The nucleotides are colored according to the type of structure that they are in. A. *farE*. B. *farR*. 
4.3 References


Appendices

Appendix 1. ASM Journals Statement of Author’s Rights.

ASM Journals Statement of Authors’ Rights

**Authors may post their articles to their institutional repositories**
ASM grants authors the right to post their accepted manuscripts in publicly accessible electronic repositories maintained by funding agencies, as well as appropriate institutional or subject-based open repositories established by a government or non-commercial entity. Since ASM makes the final, typeset articles from its primary-research journals available free of charge on the ASM Journals and PMC websites 6 months after final publication, ASM recommends that when submitting the accepted manuscript to PMC or institutional repositories, the author specify that the posting release date for the manuscript be no earlier than 6 months after the final publication of the typeset article by ASM. Please note that the information outlined above, in addition to the new author fee structure, allows authors to comply with the Policy for open access in the post-2014 Research Excellence Framework.

**Authors may post their articles in full on personal or employer websites**
ASM grants the author the right to post his/her article (after publication by ASM) on the author's personal or university-hosted website, but not on any corporate, government, or similar website, without ASM’s prior permission, provided that proper credit is given to the original ASM publication.

**Authors may make copies of their articles in full**
Corresponding authors are entitled to 10 free downloads of their papers. Additionally, all authors may make up to 99 copies of his/her own work for personal or professional use (including teaching packs that are distributed free of charge within your own institution). For orders of 100 or more copies, you should seek ASM’s permission or purchase access through Highwire’s Pay-Per-View option, available on the ASM online journal site.

**Authors may republish/adapt portions of their articles**
ASM also grants the authors the right to republish discrete portions of his/her article in any other publication (including print, CD-ROM, and other electronic formats) of which he or she is author or editor, provided that proper credit is given to the original ASM publication. ASM authors also retain the right to reuse the full article in his/her dissertation or thesis. “Proper credit” means either the copyright lines shown on the top of the first page of the PDF version, or “Copyright © American Society for Microbiology, [insert journal name, volume number, year, page numbers and DOI]” of the HTML version. For technical questions about using Rightslink, please contact Customer Support via phone at (877) 622-5543 (toll free) or (978) 777-9929, or e-mail Rightslink customer care at customercare@copyright.com.

Please note that the ASM is in full compliance with NIH Policy.

Back to Top
Appendix 2. Adaptation of *S. aureus* USA300 during growth in TSB + 50 μM linoleic acid. **A.** Cells from overnight culture grown in TSB were inoculated into TSB + 50 μM linoleic acid, to achieve OD$_{600}$ = 0.01. **B.** Cells from stationary phase of the primary culture were inoculated into fresh TSB + 50 μM linoleic acid.
Appendix 3. Differential effect of pGYlux and pGYfarE::lux on growth of *S. aureus* USA300 in TSB + 20 μM linoleic acid. Triplicate flasks of USA300 harboring pGYfarE:lux, or empty pGYlux vector were inoculated into TSB + 20 μM linoleic acid, and growth (OD$_{600}$) was monitored on an hourly basis.
Bactericidal activity of 100 µM LA towards exponential or stationary phase cells of USA300

Appendix 4. Exponential phase cells are highly susceptible to the bactericidal activity of 100 µM linoleic acid (LA). USA300 cells from an overnight culture (stationary phase) or mid-exponential phase culture (OD$_{600}$ = 0.5) were inoculated to achieve OD$_{600}$ = 0.01, in TSB containing 100 µM LA, and viability was monitored at hourly intervals. Each data point represents the mean and standard deviation of triplicate cultures.
Appendix 5. Cadmium inducible expression of farR protects USA300farR::ΦNE from the bactericidal activity of 100 μM linoleic acid. For challenge without Cd, USA300farR::ΦNE + pCNfarR was grown to mid-exponential phase in TSB + 20 μM linoleic acid, and then sub-cultured to OD<sub>600</sub> = 0.01 in TSB + 100 μM linoleic acid, followed by monitoring of viability at hourly intervals. To induce farR expression, USA300farR::ΦNE + pCNfarR was grown to mid-exponential phase in TSB + 20 μM linoleic acid and 10 μM Cd, followed by subculture into TSB + 100 μM linoleic acid and 10 μM Cd. Each data point represents the mean and standard deviation of quadruplicate flasks. Significant differences in viability at each time point were determined by unpaired one-tailed Student’s t-test; ***, P < .001; **, P < 0.01; *, P < 0.05
Appendix 6. FAR7, but not USA300, is able to grow in TSB containing 50 μM palmitoleic acid (PA). Stationary phase cells of USA300 or FAR7 were inoculated into TSB containing 50 or 100 μM palmitoleic acid, and growth (OD$_{600}$) was monitored at hourly intervals. Each data point represents the mean and standard deviation of triplicate cultures.
Appendix 7. *fakA-farER* influence virulence in a murine skin abscess model of infection. 6-week female BALB/c mice were infected with $2 \times 10^7$ CFU of USA300, USA300ΔfarER, USA300ΔfakA and USA300ΔfakA-ΔfarER. Assesses were imaged daily and surface area was quantified using ImageJ. The reduction in virulence exhibited by USA300ΔfakA-farER is significant relative to that of USA300ΔfakA as determined by a two-way ANOVA where $*P < 0.05$. Error bars represent Standard Error of the Mean. n=8 abscesses per strain.
Appendix 8. Purification of 6xHis-tagged FarR from wildtype USA300 and the variant FAR7 using nickel affinity chromatography. Cell lysate was applied onto a 1 mL His-Trap nickel affinity column that was equilibrated with binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 40 mM imidazole, pH 7.4). After washing with binding buffer, bound His-tagged protein was eluted over a linear imidazole gradient (0.1-0.5 M) in 20 mM sodium phosphate. Column fractions were assessed by SDS-polyacrylamide gel electrophoresis to check for purity. L is the unbound fraction from the column, and W is the washed flow through fraction. Purified FarR protein band is at ~ 23 kDa. A. Native FarR and B. Variant FarR7.
## Curriculum Vitae

### Education

<table>
<thead>
<tr>
<th>Period</th>
<th>Degree and Specialization</th>
<th>Institution</th>
<th>Supervisor</th>
</tr>
</thead>
<tbody>
<tr>
<td>September 2012 - Present</td>
<td>Doctorate Candidate (PhD) Microbiology and Immunology</td>
<td>Western University, London, Canada</td>
<td>Dr. Martin McGavin</td>
</tr>
<tr>
<td>September 2007 - June 2012</td>
<td>Honors Bachelor of Science (HBSc) Specialization in Biology, and Microbiology and Immunology</td>
<td>Western University, London, Canada</td>
<td></td>
</tr>
</tbody>
</table>

### Awards and Scholarships

<table>
<thead>
<tr>
<th>Year</th>
<th>Award and Scholarship</th>
<th>Institution</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>2012-2017</td>
<td>Western Graduate Research Scholarship</td>
<td>Schulich School of Medicine and Dentistry</td>
<td>Schulich School of Medicine and Dentistry</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Western University, London, Canada</td>
<td>Western University, London, Canada</td>
</tr>
<tr>
<td>2017</td>
<td>Dr. Frederick W. Luney Graduate Travel Award in Microbiology &amp; Immunology</td>
<td>Department of Microbiology and Immunology</td>
<td>Department of Microbiology and Immunology</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Western University, London, Canada</td>
<td>Western University, London, Canada</td>
</tr>
<tr>
<td>2016</td>
<td>Dr. Frederick W. Luney Graduate Travel Award in Microbiology &amp; Immunology</td>
<td>Department of Microbiology and Immunology</td>
<td>Department of Microbiology and Immunology</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Western University, London, Canada</td>
<td>Western University, London, Canada</td>
</tr>
<tr>
<td>2011-2012</td>
<td>Dean’s Honor List</td>
<td>Western University, London, Canada</td>
<td>Western University, London, Canada</td>
</tr>
<tr>
<td>2007</td>
<td>Ontario Scholar Award</td>
<td>Western University, London, Canada</td>
<td>Western University, London, Canada</td>
</tr>
<tr>
<td>2007</td>
<td>The University of Western Ontario Entrance Scholarship</td>
<td>University of Western Ontario, London, Canada</td>
<td>University of Western Ontario, London, Canada</td>
</tr>
</tbody>
</table>
Affiliations

2015- Present  The Canadian Society of Microbiologists

Publications


Manuscripts in Preparation


Invited Podium Presentations


Alnaseri, H. Identification of *farR* and *farE* as a regulator and effector of *Staphylococcus aureus* resistance to antimicrobial fatty acids. Oral presentation at the student symposium competition of the Canadian Society of Microbiologists, Regina Saskatchewan, June 16, 2015

Poster Presentations


unsaturated free fatty acids on the expression of cell surface proteins in Community-
Associated Methicillin Resistant *Staphylococcus aureus* (CA-MRSA). Poster
presentation at the Infection and Immunity Research Forum, London Ontario, November
30, 2012

**Community Involvement**

<table>
<thead>
<tr>
<th>Year</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>2017</td>
<td><strong>National Scholarship Ranking Participant</strong></td>
</tr>
<tr>
<td></td>
<td>Faculty of Science, Western University</td>
</tr>
<tr>
<td>2016</td>
<td><strong>Poster Competition Judge</strong></td>
</tr>
<tr>
<td></td>
<td>Infection and Immunity Research Forum</td>
</tr>
<tr>
<td>2016-2017</td>
<td><strong>Microbiology and Immunology Graduate Students Representative</strong></td>
</tr>
<tr>
<td></td>
<td>The Canadian Society of Microbiologists</td>
</tr>
<tr>
<td>2015-2017</td>
<td><strong>Microbiology and Immunology Graduate Students Representative</strong></td>
</tr>
<tr>
<td></td>
<td>Schulich School of Medicine and Dentistry Graduate Student Council</td>
</tr>
<tr>
<td>2012-2017</td>
<td><strong>Member of the Organizing Committee</strong></td>
</tr>
<tr>
<td></td>
<td>Infection and Immunity Research Forum</td>
</tr>
<tr>
<td>2012-2016</td>
<td><strong>Community Volunteer</strong></td>
</tr>
<tr>
<td></td>
<td>Let’s Talk Science</td>
</tr>
<tr>
<td>2012-2014</td>
<td><strong>Children's Hospital Subcommittee Member</strong></td>
</tr>
<tr>
<td></td>
<td>Strong Bones, Strong Muscles, Strong Minds</td>
</tr>
<tr>
<td>2009</td>
<td><strong>Student Leader</strong></td>
</tr>
<tr>
<td></td>
<td>Thames Valley District School Board</td>
</tr>
</tbody>
</table>